VOLUME 28 NUMBER 4

PLANT PHYSIOLOGY

OCTOBER, 1953

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PUBLISHED QUARTERLY

BY

THE AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS

AT 111 E. CHESTNUT ST., LANCASTER, PA.

Entered as second-class matter March 25, 1926, at the post office at Lancaster, Pa., under the Act of August 24, 1912.

Accepted for mailing at the special rate of postage provided for in paragraph 4, section 538. Authorized April 9, 1926.

PLANT PHYSIOLOGY

A QUARTERLY JOURNAL
DEVOTED TO ALL PHASES OF PLANT PHYSIOLOGY

Established 1926

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PLANT PHYSIOLOGY

VOLUME 28 OCTOBER, 1953 NUMBER 4

THE PHYSICAL ENVIRONMENT AND OXIDATIVE AND PHOSPHORYLATIVE CAPACITIES OF HIGHER PLANT MITOCHONDRIA

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Received December 26, 1952

Introduction

The extensive and prolonged cytological interest in the mitochondria has been complemented in recent years by an interest in the biochemical and enzymological properties of these intracellular bodies. With the recognition that the greater part of the enzymes concerned with the aerobic respiratory metabolism were intimately associated with the cell particles (**5, 11, 14**), a good deal of attention was devoted to the relation of the structure of the mitochondria to their function.

The mitochondria were observed to be discrete osmotic systems (**5, 14, 29**), and their gross structure, at least, was shown to be related to the tonicity of the environment. Fundamental alterations in the biochemical properties of the cell particles were seen to follow upon osmotically evoked physical changes (**12, 13, 14**), with the consequence that the physical integrity of the mitochondria was considered to be a prerequisite for their coordinated biochemical activity (**8**). Although the concept of structural inviolability as a requirement for organized enzymatic activity has been revised to some extent as a result of the solubilization of certain mitochondrial enzymes (**9, 34, 37**), there is still every indication that the remarkable biochemical integration of the mitochondria is closely related to their precise physical organization.

The loss of certain enzymatic functions following exposure of cellular particles to a hypotonic environment (**12, 13, 14**) has in the past been attributed primarily to the spatial disorientation of components of the mitochondrial complex (**8**). More recently, however, additional consequences of hypotonic exposure have been recognized. The cofactors of oxidation and phosphorylation have been shown to be subject to degradation by enzymes contained both in the cytoplasm and in the mitochondria themselves (**19**). The tonicity of the environment, in turn, frequently affects the effective

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concentration or activity of such degradative enzymes (**18**). In addition to the recognized consequences of hypotonic exposure mentioned above, the studies which follow describe two situations in which hypotonicity is a necessary but insufficient condition to bring about profound changes in the enzymological characteristics of the mitochondria. Both the oxidative and phosphorylative capacities of the mitochondria may be adversely affected by a low ambient tonicity. Some effort will be made below to distinguish inactivation caused by hypotonicity *per se* from inactivation brought about by other factors, yet depending upon hypotonicity as a prerequisite. The salient question underlying such studies is whether or not the coupling of oxidation to phosphorylation is obligatory in the several oxidations under consideration.

The problems investigated herein are to some extent peculiar to plant tissues. For example, the succinoxidase system, one of the most readily demonstrable oxidase systems in homogenates of animal tissues, initially proved difficult to demonstrate in leaf breis (**4**), although all presumptive evidence (**20**) indicated the enzyme to be present in intact leaves. It seemed reasonable to assume that the intermixing of vacuolar contents with the cytoplasm was in part responsible for the difficulty, as well as the failure to observe particular precautions with respect to the tonicity of the environment. Some of the evidence offered below indicates these suppositions to be correct.

Particles from various plant tissues have previously been prepared (**2, 6, 28**) which have exhibited both succinoxidase and cytochrome oxidase activity. By and large, these two systems are able to survive considerable manipulation, and it appears probable that the preparations described in the past have been suspensions of mitochondrial fragments similar to those studied by KEILIN (**17**). Recent studies (**3, 21, 26**), however, have observed the preparative requirements for the isolation of relatively intact mitochondria (**14, 36**), with the result that these studies describe plant particles capable of a much broader variety of enzymatic activity than has hitherto been reported in plant preparations.

It is more than likely that the cytoplasmic particles prepared as described in the next section do not represent a physically homogeneous cellular fraction. In particular, the work of STAFFORD (**38**) indicates that centrifugal techniques as used herein may effect rather good separation of the biochemical systems found in a plant homogenate without simultaneously producing fractions which are completely uniform when viewed in the microscope. In this text, then, the term *mitochondria* will be used in the earliest sense, as described by BENDA [see NEWCOMER (**27**)], simply to designate small threads or small grains, and will be used interchangeably with the term *particles*. A diversity of mitochondrial sizes and shapes may be expected, especially in meristematic tissue (**10**). Although perhaps no precise morphological entity is obtained by the centrifugal method as used herein, the separation of enzymological and biochemical characteristics at least reflects the existence of different functional units within the cell.

Methods

Heads of cauliflower (*Brassica oleracea*) obtained at local markets were washed with tap water, rinsed with distilled water, blotted dry, wrapped in a damp towel, and cooled in the refrigerator. The topmost 2 to 3 mm. of the immature inflorescences (referred to as cauliflower buds in the text) was then removed with the aid of a plastic grater, and the remainder of the head discarded. Buds were homogenized in a chilled mortar with approximately seven parts of water or solution, and with one-third of the bud weight reagent-grade sea-sand. All solutions were kept ice-cold, and all manipulations, including centrifugation, were carried out in a cold room maintained at 2 to 4° C. Buds were homogenized to a paste with one quarter of the requisite solution, the remaining solution being added thereafter. The pH of water or sucrose homogenates was approximately 6.6.

Ordinarily, 12 gm. of chilled buds was homogenized with 80 ml. solution and centrifuged for 5 minutes at 500 × g. The supernatants were decanted into 50-ml. plastic centrifuge tubes through a single layer of muslin and centrifuged for 20 minutes at 20,000 × g. in a Servall SS-1 angle-head centrifuge. The supernatant was siphoned off with a water pump. The precipitate was resuspended in several ml. of ice-cold water or solution with a power-driven plastic pestle which fit loosely into the centrifuge tube. The volume was then brought to 25–30 ml., and this suspension centrifuged at 20,000 × g. for 20 minutes. The precipitate from the second high-speed centrifugation was suspended in 1.0 ml. of water or solution with the plastic pestle, and was then transferred to a test tube approximately 10 mm. in diameter. With the tube held in an ice bath, the suspension was gently homogenized for 10–15 seconds with a small snug-fitting power-driven glass pestle. The volume was finally brought to 1.5 to 2.0 ml., and 0.5 ml. of this material, containing approximately 0.5 mg. protein nitrogen, was used as enzyme source in the manometer flask. Following the final high-speed centrifugation, precipitates were frequently combined, the final volume being correspondingly adjusted. The centrifugation of the initial homogenate, cleared of debris, is referred to as the first washing or first suspension. Thus, the procedure described above entails two washings. Additional washings, when specified, were carried out in the same manner as the second washing.

In each experiment an aliquot (0.2 ml.) of the final enzyme suspension was diluted 10 times, following which duplicate samples (0.8 ml.) of the diluted suspension were transferred directly to Folin-Wu tubes for micro-Kjeldahl digestion with 1.0 ml. of digestion mixture (410 mg. mercuric acetate, 35 mg. selenium dioxide, 200 ml. concentrated H_2SO_4, brought to 1.0 l. with water). Two successive additions of 2 drops of 30% H_2O_2 were provided during the digestion. The digest was diluted, Nesslerized, and the color estimated with a Klett colorimeter.

Manometric determinations were by the direct method of Warburg, and were carried out at 30° C with KOH and filter paper in the center well. Reagents were added to the manometer flasks, and the flask contents chilled

in a tray of ice before addition of the enzyme. If required, Mg^{++} or fluoride were normally added after the other reagents and the enzyme (**32**). Readings were taken at 10-minute intervals, and the $Q_{O_2}(N)$ calculated on the basis of the first two 10 minute readings.

Phosphate determinations were carried out as described by ALLEN (**1**). At zero time and at suitable intervals thereafter aliquots of the reaction mixture were mixed with equal volumes of ice-cold 2 *N* HCl. Following low-speed centrifugation of this mixture, aliquots of the supernatant were taken for the estimation of phosphate.

Coenzyme I (65% purity) was obtained from Sigma Chemical Co., adenosinetriphosphate (98–100% purity) from the Pabst Brewing Co., and cytochrome C from the Nutritional Biochemicals Corp. Hexokinase prepared from yeast was kindly supplied by Dr. J. M. Campbell. This enzyme preparation, diluted 10 times, effected the phosphorylation of 10 μmoles glucose per minute per ml. enzyme. Abbreviations used in the text and legends are as follows: Diphosphopyridine nucleotide (Coenzyme I, or Co I); Adenosinetriphosphate (ATP); 2,4-dinitrophenol (DNP).

Experimental results

OSMOTIC INJURY IN THE PRESENCE OF SOLUBLE CELL COMPONENTS

Among the numerous oxidative systems associated with the mitochondria, the succinoxidase complex has been thought to be the least fragile. Whereas certain enzymatic functions of the mitochondria are lost upon exposure of the mitochondria to a hypotonic environment (**12, 14, 33**), the succinoxidase system has long been considered able to withstand such treatment (**17, 33**). However, in an earlier study (**22**) wherein the washing of relatively intact pieces of cauliflower bud tissue was carried out at room temperature, it was observed that the succinoxidase activity of particles prepared from washed tissue was considerably less than that of particles prepared from unwashed tissue. When tissue was washed in water at 1 to 2° C or at room temperature in hypertonic sucrose, mannitol or KCl, little inactivation of succinoxidase was subsequently perceived (**22**). These observations suggested that injury due to washing was in fact a consequence of the hypotonicity of the environment, but was not to be attributed to a simple physical process (swelling) *per se*. Experiments to test these hypotheses were therefore carried out with mitochondrial suspensions.

When tissue was homogenized at 0° C in water, sufficient sucrose added to one aliquot to give a final concentration of 0.5 M and the homogenates then incubated at room temperature, no lessening of the ensuing inactivation was observed in the presence of sucrose. However, when homogenization was carried out directly in either water or 0.5 M sucrose and the succinoxidase activity of the particles prepared from each homogenate was compared, the effect of sucrose in preventing inactivation was considerable (table I). This protection was manifest whether subsequent incubation at room temperature was carried out or not. That the effect of sucrose is attributable

TABLE I

THE EFFECT OF TONICITY DURING PREPARATION AND SUBSEQUENT SUSPENSION ON MITOCHONDRIAL ACTIVITY.

Expt.	Substrate	Preparative medium (first suspension)	Second Suspension	Sucrose molarity final suspension	Tonicity in flask	$Q_{O_2}(N)$
1	Succinate	Sucrose	Sucrose			362
		Sucrose	H_2O	0.5	High	362
		H_2O	Sucrose			228
		Sucrose	Sucrose			120
		Sucrose	H_2O	0.15	Low	162
		H_2O	Sucrose			166
2	α-Keto-glutarate	Sucrose	Sucrose			220
		Sucrose	H_2O	0.5	High	89
		H_2O	Sucrose			11
		Sucrose	Sucrose			62
		Sucrose	H_2O	0.15	Low	8
		H_2O	Sucrose			0
3	Malate	Sucrose	Sucrose			284
		Sucrose	H_2O	0.5	High	144
		H_2O	Sucrose			0
		Sucrose	Sucrose			95
		Sucrose	H_2O	0.15	Low	111
		H_2O	Sucrose			58

Final concentration of reagents in manometer flasks as follows: For succinate oxidation, substrate, 2×10^{-2} M; phosphate buffer (KH_2PO_4-Na_2HPO_4) pH 7.3, 2×10^{-2} M; cytochrome c, 2×10^{-5} M. When tonicity in flask is "Low," no additional sucrose added beyond that in which enzyme (0.5 ml.) is suspended. When tonicity in flask is "High," 0.6 ml. 1.5 M sucrose added. For α-ketoglutarate oxidation, ATP and $MgSO_4$ added to final concentrations of 10^{-3} M and 1.8×10^{-3} M, respectively. For malate oxidation: As for α-ketoglutarate, plus CoI, 3.3×10^{-4} M final concentration. Final volume brought to 3.0 ml. Sucrose 0.5 M where no concentration is specified. $Q_{O_2}(N)$ indicates $\mu l. O_2$ consumed per mg. protein nitrogen per hour.

to its role as an osmotic agent is clear from the data of table II and from figure 1, wherein it is indicated that high concentrations of sucrose are required to effect a protective influence. Furthermore the succinoxidase system is protected equally well by sucrose or KCl and the malic and α-ketoglutaric oxidases, both more sensitive to preparation in water than is succinoxidase (table I), are preserved in equal measure by hypertonic solutions of sucrose, glucose or mannitol (table II).

Injury done to isolated mitochondria in a hypotonic environment was more profound than that experienced by tissue pieces, and was not completely obviated even at low temperatures. The decided thermal sensitivity of the inactivation process together with the rigorous requirement for hypertonicity at the time of homogenization suggested a possible interaction between the particles and other cytoplasmic components. Such an interaction is demonstrated in figure 2 and table III.

TABLE II

THE EFFECT OF THE COMPOSITION OF HOMOGENIZING MEDIUM ON MITOCHONDRIAL ACTIVITY.

Expt.	Substrate	Preparative medium (first suspension)	Second suspension	$Q_{O_2}(N)$
1	Succinate	H_2O		396
		KCl 2.5×10^{-4} M		537
		Sucrose	Sucrose	600
		Sucrose + KH_2PO_4 + Na_2HPO_4 (pH 7.3)		520
		Mannitol		522
2	α-Keto-glutarate	H_2O	Sucrose	90
		Sucrose	Sucrose	228
		Glucose	Glucose	234
		Mannitol	Mannitol	218
3	Malate	Sucrose		485
		Sucrose + KCl + NaCl		490
		Sucrose + KH_2PO_4 + Na_2HPO_4 (pH 6.7)	As first suspension	495
		Sucrose + KH_2PO_4 + Na_2HPO_4 (pH 7.3)		452

Final concentration of reagents as in table I, high tonicity. Sucrose, glucose, mannitol all 0.5 M. Phosphate buffer in preparative medium 0.05 M, final concentration. Salt concentration in salt-sucrose mixture, 0.075 M (1 pt. KCl: 1 pt. NaCl). Sucrose concentration lowered in buffer-sucrose or salt-sucrose mixtures to give final osmolarity of 0.5 M. Final enzyme suspension in 0.5 M sucrose.

Figure 2 indicates that exposure of the particles to distilled water for a limited time, whether at 0° or at room temperature, is in itself insufficient to cause inactivation of succinoxidase. In the presence of supernatant, however, inactivation is drastic and is much greater at the higher temperature. The irreversible action of the supernatant has been shown to be non-specific, affecting both succinic dehydrogenase and cytochrome oxidase, when measured separately, as well as malic and α-ketoglutaric oxidases. Both boiling and dialysis lessen the inhibitory action of the supernatant (table III). Although the action of the supernatant may be attributed to a combination of enzymatic and non-enzymatic causes, the possibility remains that the observed effects are brought about by a heat-labile, dialyzable substance. In any event, the agent causing inactivation is not the same as the competitive inhibitor of succinic dehydrogenase found earlier in cauliflower juice (**22**). Inhibition by the latter substance is reversible and may be overcome simply by removing the bud pieces or mitochondria from the juice.

Although succinoxidase activity is not impaired by exposure of the mitochondria to water (see below for the effect upon the concomitant phosphorylation) such treatment creates a pronounced requirement for cytochrome C (table IV) which is not due to the loss of cytochrome C from the particles, but rather to a change in its physical state (**31, 35**). Despite the resistance of succinoxidase to hypotonic washing the activity of this enzyme

complex is nonetheless dependent upon the tonicity of the environment (table I, fig. 1). When additional sucrose is withheld from the manometer flask there is little apparent difference in the succinoxidase activity of particles prepared initially in water compared to that of particles prepared initially in hypertonic sucrose (table I). It appears as if one or more components of the succinoxidase system which are sensitive to destruction by the supernatant are the same components which respond to an increase in the tonicity of the medium during the respiratory measurement.

To demonstrate the effect of the tonicity of the environment upon the oxidation of succinate, mitochondrial suspensions in 0.5 M sucrose need only

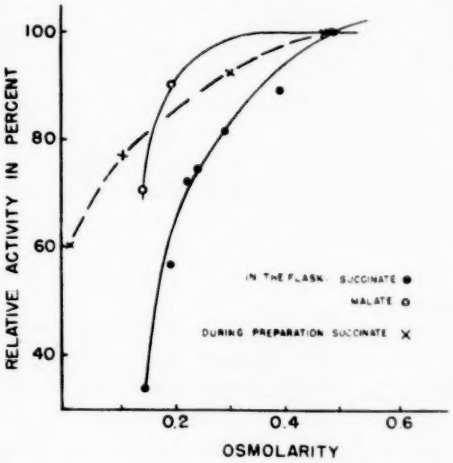

FIG. 1. The effect of the tonicity of the environment during homogenization and during the experimental period on the oxidative activity of cauliflower bud mitochondria. Final concentration of reagents as in table I, with the exception that concentration of sucrose is varied. The term *osmolarity* indicates the concentration of osmotically active components and describes the total concentration of molecular and ionic species, expressed in moles per liter. Where tonicity was varied in the flask, homogenization was carried out in 0.5 M sucrose, and where tonicity was varied during the preparation, the final osmolarity in the flask was in each case 0.5 M.

be transferred to manometer flasks containing varying quantities of sucrose. With malate as substrate, however, no stimulation of oxidation was observed upon addition of sucrose to the flask unless the enzyme aliquot contained something less than 250 μmoles sucrose (0.5 ml. of 0.5 M sucrose). Thus when 0.5 ml. enzyme suspended in 0.15 M sucrose (75 μmoles) is introduced into the manometer flask, the addition of as little as 175 μmoles sucrose will bring about near maximal rates of malate oxidation (table V, fig. 1). There appears to be no apparent injury to the malic oxidase system following passage of the particles through a small volume of 0.15 M solution.

Table I describes the effect upon succinoxidase, malic oxidase, and α-ketoglutaric oxidase, of preparation of the mitochondria in water, of pas-

TABLE III

THE EFFECT OF HEAT-TREATED OR DIALYZED SUPERNATANT ON MITOCHONDRIAL SUCCINOXIDASE.

Incubation medium	$Q_{O_2}(N)$	Per cent. control
H_2O	710	100
KCl 2×10^{-2} M	590	83
Supernatant	322	45
Boiled supernatant	500	70
Dialyzed supernatant	520	73

Final concentration of reagents as in table I, high tonicity. Substrate, succinate. Particles prepared in 0.5 M sucrose. Separately prepared water homogenate dialyzed at 2°C overnight or heated for 5 min. at 90° to 100°C. Dialyzed, boiled, and untreated water homogenate all cleared before use by centrifugation at 50,000 × g. for 45 min. in refrigerated Spinco. Incubation period 30 min. at room temperature. Particles then sedimented at 0°C and resuspended in 0.5 M sucrose.

sage of the mitochondria through a hypotonic phase, and of the tonicity of the environment during the respiratory measurement. Preparation in water is in each case deleterious, whereas passage through a hypotonic phase is apparently without consequence with respect to succinoxidase activity, but of considerable consequence with respect to both malic and α-ketoglutaric oxidase activity. The tonicity in the flask is in each case an important determinant of the final oxidative rate, and the tonic requirements vary with the different oxidases.

It will be shown below that addition of dilute salt to hypotonic bathing media may preclude injury to the malic and α-ketoglutaric oxidases, when such injury would be manifest following suspension of the mitochondria in distilled water. However, preliminary experiments established the fact that dilute salt does not obviate the damage done by initial homogenization in distilled water. Furthermore, the addition of salt or of buffer is without effect when supplied in addition to sucrose during the initial grinding (table II), in distinction to the observations of MILLERD (**25**) with mitochondrial preparations from mung beans. The susceptibility of malic and α-ketoglutaric oxidase to hypotonic treatment is not related to the requirement of these systems for exogenous adenylates or pyridine nucleotides (**21**), for these oxidases have been shown to respond to an increase in tonicity whether or not additional cofactors were provided.

OSMOTIC INJURY IN THE ABSENCE OF SOLUBLE CELL COMPONENTS

Since one or more soluble protoplast components were shown to bring about the inactivation of several mitochondrial oxidases under hypotonic conditions, those cases were reexamined which showed impairment of oxidase activity following exposure to distilled water (table I), to determine whether residual supernatant was responsible for the observed inactivation. To test this possibility, particles were twice centrifuged in 0.5 M sucrose prior to a third centrifugation in water. Particles so prepared were compared to mitochondria undergoing hypotonic washing during the second

centrifugation, as usual, followed by a third suspension and centrifugation in 0.5 M sucrose. Surprisingly the inactivation caused by the first sequence above was always considerably greater than that caused by the second (table VI, Experiment 1). Residual supernatant was therefore ruled out as the cause of inactivation.

Further experiments indicated that the relative advantage of the second sequence was not to be explained in terms of recovery of the particles in hypertonic solution following exposure to water. If the first washing series was modified so that the third suspension was carried out in a small volume of water (2.0 ml.) instead of in the usual large volume (30 ml.), the inacti-

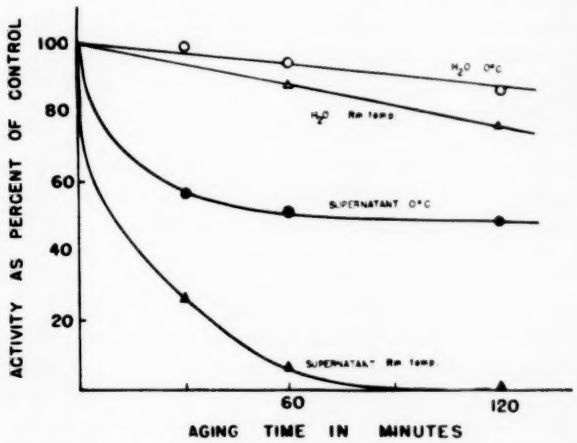

FIG. 2. The relative effect of distilled water and protoplast components on cauliflower succinoxidase. Final concentration of reagents as in table I. Particles aged in water were prepared in 0.5 M sucrose. Where treatment with supernatant is indicated, particles were prepared from aged water homogenates. Controls prepared in sucrose, and activity measured at once.

vation normally caused by water washing was sharply reduced (table VI, Expt. 2).

It appeared unlikely that the small quantity of sucrose to be found in the precipitate following the second centrifugation afforded osmotic protection when this precipitate was resuspended in a small volume of water, since the washing of particles in a large volume of 0.15 M sucrose was almost as detrimental to malic oxidase as washing in a large volume of water. An explanation was therefore sought in terms of the dilution during large volume washing of some factor necessary to the integrity of the mitochondrial enzymes in question.

When a small-volume water suspension of mitochondria was centrifuged and the supernatant removed, the particles were subsequently active in the oxidation of malate, whereas if a similar suspension was dialyzed they were not. Thus injury was attributed to hypotonicity in the absence of certain

TABLE IV

THE CYTOCHROME C REQUIREMENT OF MITOCHONDRIAL SUCCINOXIDASE.

Homogenizing medium	Resuspending medium	$Q_{O_2}(N)$		Per cent. increase due to cytochrome
		− Cytochrome c	+ Cytochrome c	
H_2O	Sucrose	84	250	198
Sucrose	H_2O	109	398	265
Sucrose	Sucrose	313	398	27

Final concentration of reagents as in table I, high tonicity. Succinate as substrate. Final suspension of particles in 0.5 M sucrose in all cases.

soluble components normally present in a mitochondrial suspension. Neutral salts were first tested for possible identity with the soluble factor in question, simply because it was reasonable to envisage the presence of such salts in bud homogenates, and to consider that such salts would be readily susceptible to being washed away. When particles were suspended in as low as 10^{-3} M KCl instead of in water during the third centrifugation (following two centrifugations in 0.5 M sucrose), inactivation of both malic and α-ketoglutaric oxidase was largely precluded (tables VI and VII). This sparing action appears to be unspecific insofar as the potassium or sodium salts of chloride or phosphate are equally effective (table VI). In but one or two instances the suspension of particles in neutral salt in small volume has proved deleterious, and these aberrations remain unexplained.

In the experiments described above, hypotonicity *per se* was not sufficient to bring about the observed inactivation in relatively short incubation periods. For damage to occur, hypotonicity must be imposed in the absence of low levels of inorganic salt. This explains why hypotonic treatment following the first centrifugation in sucrose is not as devastating as similar treatment following two hypertonic washings. In the latter instance the salt level has been more rigorously depleted before the subsequent exposure to

TABLE V

THE RELATION OF MALATE OXIDATION TO AMBIENT TONICITY.

Experiment	Final suspension[1] (2.0 ml.)	Sucrose addendum to flask (millimoles)		
		0.0	0.175	0.9
1	0.5 M sucrose	485	....	483
	H_2O	258	....	385
	0.01 M KCl	345	....	459
2	0.5 M sucrose	350	....	372
	0.15 M sucrose	283	338	376
3	0.15 M sucrose	283	394	417

[1]First and second suspensions in 40 and 30 ml. 0.5 M sucrose respectively.

Reagent concentrations as in table I, high tonicity, with l-malate as substrate. In addition, glucose, 10^{-2} M final concentration [as substrate for endogenous hexokinase (21)].

TABLE VI

THE EFFECT OF DILUTE SALT ON THE OSMOTIC INACTIVATION OF MALIC OXIDASE.

Experiment	Second suspension	Third suspension[1]	$Q_{O_2}(N)$
1	Sucrose	Sucrose	570
	H_2O	Sucrose	392
	Sucrose	H_2O	119
2	Sucrose	Sucrose	316
	Sucrose	H_2O	46
	Sucrose	Sucrose (2.0 ml.)	458
	Sucrose	H_2O (2.0 ml.)	324
3	Sucrose	Sucrose	458
		H_2O	218
		KCl[2]	430
		NaCl	464
		k phosphate, pH 7.0	434
		Na phosphate, pH 7.0	487
4	Sucrose	H_2O	123
		4×10^{-4} M KCl	181
		2×10^{-3} M	294
		1×10^{-2} M	362
		5×10^{-2} M	360

[1]Volumes of all suspensions 30 ml, except where specified.
[2]Salts 2×10^{-3} M.

Reagent concentration as in table I, high tonicity, with l-malate as substrate. In addition, glucose, 10^{-2} M final concentration. First and final suspension in 0.5 M sucrose in all cases. Final suspension 2.0 ml.

distilled water. Hypotonicity is clearly a prerequisite to damage of the particles. Osmotic injury may conceivably follow the exposure of normally protected areas of the mitochondrion to the environment, or may be the consequence of leaching of ions which in hypertonic solution would not be washed away. Whatever the process, it appears irreversible. If the mitochondria are distended in water and dilute salt is then added before the suspension is made hypertonic again, damage is not diminished.

THE RELATIVE EFFECTS OF HYPOTONICITY ON OXIDATION AND PHOSPHORYLATION

Numerous reports (**23, 32, 33**) have indicated that the oxidative and phosphorylative capacities of mitochondria are susceptible in different degree to inactivation by hypotonic treatment. In view of the above findings that hypotonicity *per se* frequently failed to bring about the impairment of oxidative activity, it was deemed interesting to investigate the effect of hypotonicity on both oxidation and phosphorylation under conditions where hypotonicity was achieved both in the presence and absence of neutral salts.

Following two washings in 0.5 M sucrose in the centrifuge, particles were suspended in a large volume (25–30 ml.) of water or 5×10^{-3} M KCl, and

centrifuged again. The oxidative and phosphorylative capacities of particles so treated were compared to the corresponding activities of particles washed for the third time in sucrose-KCl (table VII). It is interesting that whereas the oxidation of succinate is affected but to a limited extent by submitting the particles to a large volume of distilled water, the concomitant

TABLE VII

THE EFFECT OF HYPOTONICITY ON OXIDATION AND PHOSPHORYLATION AS RELATED TO THE SALT CONTENT AND VOLUME OF THE SUSPENDING MEDIUM.

Expt.	Substrate	Third suspension[1] (25 ml.)	Final suspension (2.0 ml.)	$Q_{O_2}(N)$	P/O
1	α-Ketoglutarate	Sucrose-KCl		179	1.48
		H_2O	Sucrose	42	1.11
		KCl		159	1.19
2	α-Ketoglutarate	Sucrose-KCl		174	1.43
		H_2O	Sucrose	57	0.81
		KCl		167	1.63
	Succinate	Sucrose-KCl		234	1.42
		H_2O	Sucrose	197	0.0
		KCl		228	0.42
3	Malate	....	H_2O, incubated	337	1.55
		....	Sucrose, incubated	348	1.59
		H_2O	Sucrose	171	1.05
		KCl	Sucrose	281	1.07
4	Succinate	....	H_2O, incubated	183	0.0
		....	Sucrose, incubated	279	1.34
		H_2O	Sucrose	117	0.0
		KCl	Sucrose	119	0.62
5	α-Ketoglutarate	....	H_2O, incubated	179	2.36
		....	Sucrose, incubated	220	2.16
		H_2O	Sucrose	59	0.77
		KCl	Sucrose	162	2.03

[1]Sucrose 0.5 M, KCl 10^{-3} M.

Reagent concentrations as in table I, high tonicity. In addition, glucose, 10^{-2} M final concentration; NaF, 10^{-2} M; and yeast hexokinase (sufficient to phosphorylate 0.5 μmole glucose minute). Sufficient 1.5 M sucrose added to water suspensions following pre-experimental incubation to make final concentration 0.5 M. Incubation 20 min. at 0°C where indicated. P/O ratio indicates μmoles inorganic phosphate esterified per μatom oxygen utilized.

phosphorylation is completely abolished. The presence of neutral salt exerts some small protection of the largely refractory oxidative system and very little protection of the phosphorylative system. With α-ketoglutarate as substrate, however, the inactivation of the oxidative system caused by passage of the particles through a large volume of water is considerable, and the sparing action of neutral salt is also large. Although phosphorylation

is diminished to some extent by water treatment, the diminution is but slightly in excess of that exhibited by the respiration, and there is evidence of sparing or protection of the phosphorylative machinery by neutral salt.

Hypotonicity in small volume was shown to have but a limited influence upon respiration (table VI). In experiments 3 to 5 of table VII the effect upon oxidation and phosphorylation of exposure of particles to small volumes of water is compared directly to the effects of large volume washing in water or dilute salt. Neutral salt protects both the oxidative and phosphorylative systems when α-ketoglutarate is given as substrate, whereas with malate a greater sparing action is exerted upon the oxidative system. The phosphorylative mechanisms associated with succinate oxidation appear to be particularly sensitive to hypotonicity, being made completely inoperative by both small and large volumes of distilled water, and being spared but little by neutral salt. Small-volume exposure to distilled water appears to exert but a limited effect upon the oxidative and phosphorylative characteristics of malic and α-ketoglutaric oxidase.

Discussion

In much of the work relating mitochondrial structure to function (**12, 13, 14**) the inactivation of certain mitochondrial enzyme systems brought about by hypotonic environments has been attributed neither to leaching nor to dilution effects, but rather to the alteration of requisite spatial configurations. CLAUDE (**5**) has suggested the existence of a mitochondrial membrane which may be modified or even disrupted in a hypotonic environment. Although the existence of such a membrane has recently been reaffirmed by excellent electron microscope studies (**30**) much evidence militates against the view that effects upon the membrane are causal to the gross enzymatic changes observed.

The experiments presented above have suggested that under certain conditions the swelling of the mitochondria in hypotonic solution may not in itself be responsible for injury to the oxidative and phosphorylative systems, but may be a prerequisite to such injury. At least two distinctly different destructive effects have been described for which hypotonicity is necessary but not sufficient. A type of injury at least in part non-enzymatic is indicated which is attributable to the action of one or more soluble components of the protoplast upon several of the mitochondrial enzymes. Succinic dehydrogenase, cytochrome oxidase, malic and α-ketoglutaric oxidases, and succinoxidase are all susceptible to this type of inactivation. The effect upon malic, α-ketoglutaric, and succinic oxidase is in each case greater than can be accounted for by the effect upon the cytochrome oxidase system. This type of inactivation is largely precluded by carrying out the initial homogenization of tissue in 0.5 M sucrose, in the cold. The instantaneous injury suffered by the mitochondria when cauliflower buds are homogenized in water indicates the osmotic response of mitochondria to be even more rapid than previously suggested (**29**) and clearly distinguishes this type of

injury from the irreversible changes effected by hypotonic exposure in excess of fifteen minutes (**12**).

In an early study of the effect of tonicity upon certain oxidative systems, POTTER (**31**) described a situation wherein rat liver cells washed isotonically and then exposed to distilled water were able to oxidize octanoate, whereas cells first exposed to distilled water and then washed were not. This anomaly was interpreted in terms of the removal of an unknown necessary factor by washing following lysis. However it may well be that the situation is comparable to that found here with cauliflower preparations.

When cauliflower mitochondria are once removed from the supernatant solution following centrifugation, the succinoxidase activity is shown to be little affected by exposure to distilled water. The malic and α-ketoglutaric oxidase activity also survives such exposure when suspension of the mitochondria is done in small volume. When exposure to distilled water is carried out in larger volume, succinate oxidation is still not greatly affected, whereas the oxidation of malate and α-ketoglutarate is appreciably diminished. However, if neutral salt in concentration as low as 1 to 5×10^{-3} M is substituted for water, this inactivation is largely precluded.

Although hypotonicity has been considered a sufficient condition to evoke enzymatic changes within mitochondria, CLAUDE (**5**), in studying the effect of the ambient tonicity on the structure and chemical constitution of the large granules (primarily mitochondria) of guinea pig liver, has made the distinction between hypotonicity *per se* and hypotonicity in the absence of salts. The latter condition was observed to bring about the immediate disintegration of the large granules which otherwise were seen to change their structure more slowly. The comparative impunity with which small-volume washing of cauliflower mitochondria may be effected, observed here with respect to enzymological properties, is comparable to the structural immunity of the large granules observed by Claude under similar conditions. That qualitatively distinct types of structural changes may occur, and that these changes may variously affect the enzymatic characteristics of the mitochondria, has been emphasized by HARMAN (**12**), although no consideration was given in the latter work to the absence of salt in an environment of low osmotic pressure as a condition distinct from hypotonicity *per se*. It will be of interest to learn whether the salt requirement is for the integrity of the particles only, or whether the requirement is exhibited as well by the solubilized systems (**9**).

The phosphorylative and oxidative characteristics of cauliflower bud particles are differently affected by hypotonicity. The phosphorylations accompanying succinate oxidation are completely obviated by hypotonic treatment both in small and in large volume, and little protection of this phosphorylative activity is afforded by neutral salt. The phosphorylation related to α-ketoglutarate oxidation is destroyed only by large volumes of distilled water, and this destruction is precluded by neutral salt. Phosphorylation accompanying malate oxidation, in turn, appears to be in an

intermediate category, being sensitive to exposure to large volumes of distilled water, yet incompletely protected by neutral salt.

Although particles may obviously begin to swell within five minutes when placed in hypotonic medium (**29**), irreversible injury to the cyclophorase system of rabbit liver has been considered to be a consequence of hypotonic exposure in excess of fifteen minutes (**12**). It is significant that in the presence of salt the oxidative and phosphorylative systems concerned with malic and α-ketoglutaric acid metabolism are unaffected by exposures in excess of 15 minutes, although the effect upon the cyclophorase system in its entirety has not been studied.

It is not suggested that in the experiments above the extent to which a process is disrupted by exposure of the mitochondria to hypotonic medium has any quantitative significance. That is, the extent of the inhibition of respiration or phosphorylation may well have been either greater or less depending upon variations in temperature, duration of exposure and perhaps upon the biological material. However, the qualitatively different behavior of the succinoxidase system compared to the α-ketoglutaric and malic oxidase systems seems significant. That is, the interesting situation obtains wherein the succinoxidase system is almost insensitive to exposure to a hypotonic environment, whereas the associated phosphorylative system is completely destroyed by such a procedure. On the other hand, malic and α-ketoglutaric oxidase are both sensitive to a hypotonic environment and the associated phosphorylative systems are susceptible to approximately the same extent as the oxidative processes. It would appear that the succinoxidase activity does not depend upon concomitant phosphorylation, whereas malic and α-ketoglutaric oxidase activity does.

More than one type of phosphorylative mechanism is indicated. Furthermore it would seem that more than one of the phosphorylations accompanying malate or α-ketoglutarate oxidation occur at loci not shared with the succinoxidase system, since the P/O ratios observed during the oxidation of either malate or α-ketoglutarate, by particles previously suspended in small volumes of distilled water, are well above 1.0, whereas the same ratio for succinate oxidation under similar circumstances is zero. Some indication of the existence of more than one type of phosphorylative mechanism has already been given by HUNTER (**16**) who showed that the phosphorylation accompanying the primary oxidation of α-ketoglutarate is insensitive to 2,4-dinitrophenol (DNP), whereas the phosphorylations accompanying electron transfer from reduced coenzyme I onward are DNP sensitive.

In all probability the P/O ratios obtained in the foregoing studies are lower than could be expected. The experimental values suggest a reasonable probability of 2.0 for succinic oxidation and 3.0 for malic and α-ketoglutaric acid oxidation. The P/O ratio for α-ketoglutaric acid oxidation may be even greater than 3.0 (**16**). Thus, in the studies described above, some part of the phosphorylative system is already inoperative at the time of the measurement as a result of the preparative technique, and it is impossible

to accurately describe quantitatively the effect of the imposed environmental treatment on the various phosphorylative systems.

Bonner and Millerd (3) have carried out extensive studies pertaining to the ability of mitochondria prepared from mung beans to effect oxidative phosphorylation. The P/O ratios obtained in the latter studies have been predominantly close to 1.0, although occasional experiments have yielded ratios close to 3.0 for the oxidation of α-ketoglutarate. In view of the variable lability of the phosphorylative systems described in the experiments presented here, and the occasional high ratios observed by the above authors, it seems likely that ratios in mung beans will ultimately prove to be above 1.0. Mung beans perhaps represent a material in which uncoupling is more readily effected during enzyme preparation than is the case with cauliflower buds.

When particles have suffered no irreversible injury during the preparation or washing, the activity of the mitochondrial enzymes is dependent upon the tonicity in the manometer flasks. Similar observations have been made by others (7, 24). The tonicity requirements appear different for the oxidation of different substrates and, interestingly, succinoxidase, the oxidative system least sensitive to hypotonic treatment during the preparation, responds most perceptibly to an increase of tonicity in the flask. In spite of the fact that the effect of the ambient tonicity upon mitochondrial function is frequently indirect, there is compelling evidence that the tonicity *per se* is of great consequence, both with respect to maintaining certain phosphorylative systems intact, and with respect to the achievement of maximal oxidative activity.

Summary

The effect upon cauliflower bud mitochondria of preparation in water, of passage through a hypotonic phase, and of the tonicity of the environment during the experimental period has been investigated with respect to the oxidative and phosphorylative capacities of the succinoxidase, malic oxidase, and α-ketoglutaric oxidase systems.

Two types of enzymatic inactivation have been described for which hypotonicity is a necessary but insufficient condition. The exposure of cauliflower bud mitochondria to a hypotonic environment in the presence of soluble components of the protoplast results in the rapid and irreversible deterioration of the malic, α-ketoglutaric and succinoxidase systems. Such destruction may be prevented by the preparation of mitochondria in cold hypertonic solution.

When mitochondria are suspended in distilled water, in volumes commonly employed, in the absence of other protoplast components, the succinoxidase system remains unaffected, whereas the malic and α-ketoglutaric oxidase systems suffer considerable injury. This type of injury is minimized or precluded either by the substitution of dilute neutral salt solutions for distilled water, or by suspension of the mitochondria in small volumes of distilled water.

The phosphorylative system in association with the succinoxidase complex is completely destroyed by any exposure to a hypotonic environment, whether or not neutral salts are present. On the other hand the phosphorylative machinery in association with malic and α-ketoglutaric oxidase is affected to approximately the same extent as the oxidative systems by hypotonic exposure, and both the oxidative and phosphorylative systems are protected by neutral salt. The different effect of low tonicity upon the P/O ratios observed with various substrates has led to the suggestion that more than one of the phosphorylations accompanying malate or α-ketoglutarate oxidation occur at loci not shared in common with the succinoxidase system.

The author wishes to express his gratitude to the Rockefeller Foundation under whose auspices this work was initiated during the tenure of a Rockefeller Foundation Fellowship in the Biological Sciences at Cambridge and Sheffield Universities in 1949–1950.

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THE ABSORPTION AND TRANSLOCATION OF IRON

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Received August 28, 1952

Introduction

Iron has been recognized as an essential element for quite some time, yet much of the data concerning the uptake and translocation of this element by plants is conflicting or incomplete. Three factors have emerged, however, as being highly influential in the process of iron absorption. These factors are pH, phosphorus concentration, and the chemical form of the iron.

OLSEN (**7**) has indicated that high pH and high phosphorus concentration of the nutrient solution induce iron deficiency chlorosis. He also suggested that too high a phosphorus concentration could cause a precipitation of iron along the veins of the leaf. FRANCO and LOOMIS (**2**) somewhat more recently have shown that iron absorption from cultures is reduced by phosphorus, especially at pH 6 or higher. The effect of various forms of iron on iron uptake has been investigated in a relative sense, the data being concerned primarily with those forms of iron giving the best growth under a given set of conditions.

Iron has long been commonly referred to as a non-mobile element. Indeed, GILE and CARRERO (**4**) at a very early date conducted an experiment with rice which demonstrated this immobility. However, since that time, the work on iron translocation has been sparse and incomplete.

The present paper is concerned with those factors which have been shown to effect the absorption and translocation of iron, namely, pH, phosphorus concentration and the amount and chemical form of the iron used. An attempt has been made to define the effects of these factors in a quantitative sense and to determine interrelations between them.

Materials and methods

Red Kidney bush beans (*Phaseolus vulgaris* L.) were used as the experimental plants throughout this investigation. They were obtained from Burpee's standard commercial stock.

The plants were cultured in 6-liter enameled refrigerator pans according to the technique outlined by BIDDULPH (**1**). The culture period in the refrigerator pans normally lasted 12 days. The nutrient solution used consisted of the following salts and concentrations: $Ca(NO_3)_2$ 0.0025 M, KNO_3 0.0025 M, $MgSO_4$ 0.0010 M, KH_2PO_4 as indicated for each experiment,

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H_3BO_4 0.5 p.p.m. B, $CuSO_4$ 0.02 p.p.m. Cu, $MnCl_2$ 0.5 p.p.m. Mn, $ZnSO_4$ 0.05 p.p.m. Zn and Fe as indicated for each experiment. During the first 4 days nutrient solution of half strength was used. At the beginning of the next 4-day period, the solutions were changed to full strength and 0.5 p.p.m. of iron was included. The solutions were again changed for the final 4-day period, full strength solutions again being used. The various experimental conditions were produced in the nutrient solutions during this last 4-day period.

At the conclusion of the final 4-day experimental period the plants were harvested. Those upon which analyses were to be made were divided into trifoliate leaves (blades only), primary leaves, stems (and petioles), and roots. The separated material was then placed in an oven at 70° C to dry. That material used for autoradiographs was pressed between newspaper folds and quickly dried in an oven at 70° C.

After the plant material had dried for at least 2 days, the ovens were turned off and allowed to reach room temperature. The plant materials were then removed, weighed, and carefully placed in 200-ml. Kjeldahl flasks through smooth-surfaced paper funnels. Digestion of plant parts was carried out as a modification of a method described by PIPER (**8**) using sulfuric and perchloric acids. After cooling, the contents of the flasks were quantitatively transferred to 250-ml. volumetric flasks. Suitable aliquots were then removed for analysis.

Total iron was determined by a modification of the thiocyanate method of SANDELL (**11**). The color was developed under standard conditions and the transmittance was determined with a Ceneo Photelometer using a 10-ml. cell 1 cm. thick and a green filter. The readings were compared with a curve prepared from a set of standards made up according to the same procedure as the unknown samples, including the digestion.

It has been found in working with radioiron that the isotope Fe^{55} offers numerous advantages for this type of work over the better known isotope Fe^{59}. Among these advantages are the longer half-life necessitating fewer decay corrections, the greater specific activities obtainable, and the lower energies of the radiations eliminating the necessity for extra shielding. Therefore, the tracer work to follow was done with this isotope. The stock solution of iron was prepared by adding a tracer amount of Fe^{55} to a solution of $FeCl_3$ of known concentration. This solution will hereafter be referred to as Fe^*Cl_3. The resulting activity of Fe^{55} in the nutrient solution was 0.1 μc./l.

In preparation of the samples for radioiron determination, use was made of a technique (**9**) similar to the thiocyanate method described for total iron. In this case, however, the ferric thiocyanate complex was extracted with isoamyl alcohol and an aliquot evaporated on a stainless steel counting disc. The discs were then placed in a Nucleometer (Radiation Counter Laboratories) and "counted." By utilization of a previously prepared relationship between activity and total iron in the radioactive solution, the marked iron content of the unknown samples could be determined.

The translocation studies were carried out by a phloem injection technique described by BIDDULPH (1). The radioiron solution which was injected into the second trifoliate leaf from the base assayed 0.46 mc. of Fe^{55}/ml. and contained 1000 p.p.m. of carrier iron as $FeCl_3$.

After harvest, autoradiographs of the injected plants were prepared to determine the extent of translocation. This was done by placing the pressed and dried plants directly on a 10×12 inch sheet of Eastman no-screen x-ray film. These were placed in an x-ray exposure folder and allowed to expose for 15 days. The films were then developed. The numerous steps in exposure, development and reproduction were maintained uniform throughout so that semi-quantitative comparisons between autoradiographs might be made.

Results and discussion

THE NUTRIENT RELATIONSHIP OF VARIOUS IRON COMPOUNDS

In order to complete an effective study of iron uptake from a nutrient medium, it was considered necessary to have an appreciation of the of the reaction of various types of iron compounds in the nutrient solution to be used. Therefore, a series of experiments was conducted whereby the rate of formation of iron precipitates could be followed.

One liter of nutrient solution of a composition previously indicated containing 0.0005 M phosphorus was placed in a flask. To this was added 1.0 p.p.m. of iron as the particular compound being studied. The pH was adjusted to the desired level with *N* KOH or H_2SO_4, and the flask was covered and placed in darkness at room temperature. Samples of the supernate were subsequently taken at 1-, 3-, and 7-day intervals by pipetting from the top of the solution. The solutions were otherwise undisturbed so that the aliquot contained soluble iron as well as any particles of precipitate small enough to remain in colloidal suspension. The aliquots were reduced in volume by evaporation and the iron content determined by the thiocyanate method.

The results are indicated in figure 1. It will be noted that ferric phosphate is very insoluble and the solubility is little affected by pH. Ferric chloride and ferric nitrate are very similar in reaction. They too become quite insoluble and are not influenced appreciably by pH. The organic salts of iron—that is, oxalate, tartrate, and citrate—are very similar in that they are somewhat more soluble at a pH of 4.0 than pH 7.0, making their use practicable at a lower pH This increase in solubility over the inorganic salts may be due to complex formation with the organic anion. Ferric humate (5) was found to be the most promising iron compound to use in experiments conducted over a wide pH range because of its constant and relatively high solubility. Ferrous sulfate was found to be unique in its reaction to pH being relatively soluble at pH 4.0 but quite insoluble at pH 7.0 with corresponding gradations between. Both potassium ferrocyanide and potassium ferricyanide were included in the above experiment, but no

precipitation of these compounds was found within the range of experimental conditions imposed.

As a part of this experiment, 10 groups of plants were grown in nutrient cultures each group replicated once. Each culture incorporated one of the various forms of iron as the source of this element. After 6 days in the

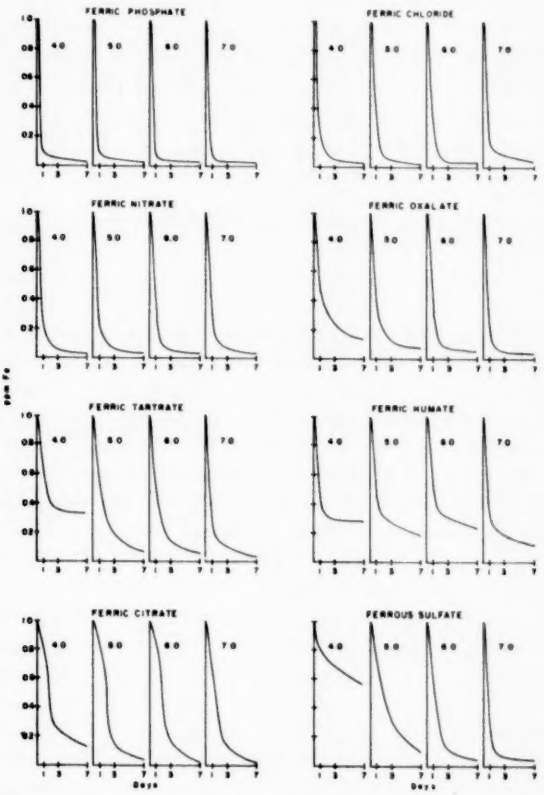

FIG. 1. The amount of iron remaining in solution at various pH values during time intervals of one, three, and seven days. Ten forms of iron were employed, two forms explained in text. Phosphorus concentration of the solution was 0.0005 M.

solutions the plants were removed and the various parts subjected to an analysis for total iron. Figure 2 presents the results obtained.

It can be seen that the amount of iron associated with the roots varies with the compound introduced into the nutrient solution. It is also apparent that the concentration of iron in the leaves is independent of the solubility of the iron compound employed. Finally, there is no apparent dependence of the concentration of iron in the leaves on the concentration of iron associated with the roots.

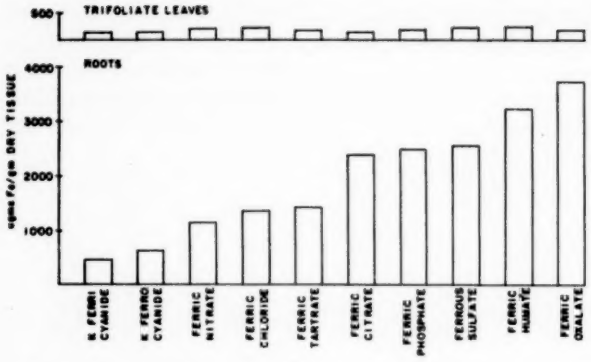

FIG. 2. The effect of ten iron compounds on the absorption of iron at pH 4.0. The phosphorus concentration was 0.0005 M

THE EFFECT OF pH ON IRON UPTAKE

The uptake of iron from a nutrient solution is considerably influenced by changes in pH. As later evidence will indicate, it is quite probable that this influence is exerted through an increased mobility of iron as well as an increase in adsorption on the root surfaces at low pH values. Table I tabulates the data of an experiment conducted to show the effect of pH on iron uptake. It is clearly seen (fig. 3) that as the hydrogen ion concentration increases the accumulation of iron associated with the roots also increases. Likewise, the uptake into the trifoliate leaves increases. This relationship is found when 1.0 p.p.m. of iron is present as ferric nitrate. A similar relationship is found when ferric humate is used (**10**).

It is not precisely understood why the accumulation of iron associated with the roots in solution culture should increase as the hydrogen ion concentration increases. This phenomenon is undoubtedly associated with the type of particle formed in the precipitation of the iron, since nearly all of the iron becomes insoluble at those pH levels studied. A good portion of the precipitate formed will be of the ferric oxide type. Ferric oxide particles are pH sensitive in formation, the rate of coagulation being particularly influenced. Variations in the rate of coagulation can result in the formation of crystalline hematite, gelatinous hydrated ferric oxide particles or the suspended hydrosol itself when coagulation is at a minimum. It is postulated that the increase in iron concentration on the roots as the hydrogen ion concentration increases is due to the difference in the ability of these various types of iron particles to adsorb on the roots. It is assumed that most of the iron associated with the roots under the above conditions is adsorbed iron. The very high concentrations found, about 10 times that of the trifoliate leaves, indicate that it is not true absorption. Such abnormal concentration ratios of root over leaves would not be expected if all the iron associated with the roots in this case was absorbed iron.

In those samples where no iron was added to the nutrient solution, the principal source of iron was from the cotyledons (**12**). There was also approximately 0.004 p.p.m. in the nutrient solution added as impurities in the salts. Under these conditions chlorosis readily occurred at pH 7.0 and 6.0, rarely at 5.0 and never at 4.0. This indicates that at pH 4.0 more iron from the cotyledons was utilized by the plants. This fact is also evidenced by the slightly higher content of iron in the tissues at the lower pH values. In referring to the data of table I it is found that a slightly greater amount of iron is found in the spent nutrient at the lower pH values evidently having

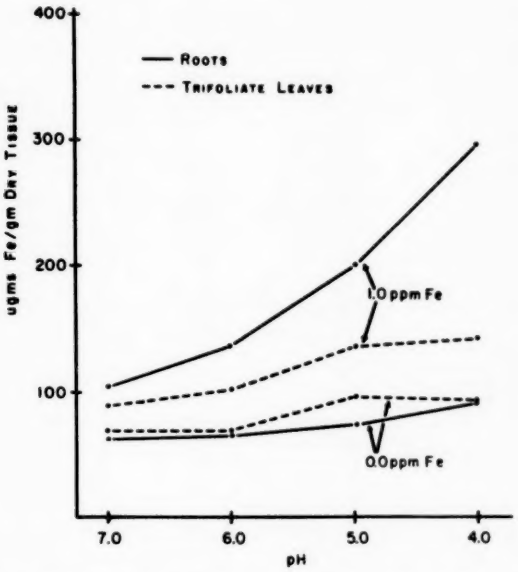

FIG. 3. The effect of pH on uptake of iron from a nutrient solution. Ferric nitrate was used as the source of iron for that series having iron. The phosphorus concentration was 0.005 M. The concentration of iron on the roots of the 1.0 p.p.m. series should be multiplied by ten.

leached from the plant. This could be caused by an increased mobility of iron or a difference in membrane permeability with varying pH.

INFLUENCE OF IRON CONCENTRATION ON IRON UPTAKE

The concentration of iron in the nutrient solution does not appear to have a proportional effect on the uptake of this element over the range studied. Figure 4 indicates that in general the uptake increased as the concentration increased, but the greatest rate of uptake only doubled when the concentration of iron was increased as much as 10 times. At the lower concentrations of iron (0.004 to 0.1 p.p.m.) the rate of increase of iron uptake is very small regardless of the pH. This can probably be attributed to the

TABLE I

THE EFFECT OF PH ON UPTAKE AND GROWTH AT TWO IRON LEVELS USING FERRIC NITRATE. THE UPTAKE IS EXPRESSED AS μGM. FE/GM. OF DRY TISSUE. THE DRY WEIGHT IS THAT OF SIX PLANTS. THE DUPLICATES SHOWN ARE THOSE OF TWO DIFFERENT PANS.

		p.p.m. Fe in spent nutrient solution	Primary leaves		Trifoliate leaves		Stems		Roots	
			Dry wt. gm.	Fe Conc.	Dry wt. gm.	Fe Conc.	Dry wt. gm.	Fe Conc.	Dry wt. gm.	Fe Conc.
	pH 4.0	.15	1.69	375	1.16	159	1.27	51	.69	2970
		.15	1.58	163	1.16	123	1.09	40	.70	2920
	pH 5.0	.29	1.74	174	1.47	114	1.23	34	.76	2000
		.37	1.80	316	1.52	153	1.30	45	.73	1200
1.0 p.p.m. Fe										
	pH 6.0	.42	1.76	141	1.44	99	1.30	30	.75	1280
		.37	1.73	122	1.52	100	1.29	30	.74	1430
	pH 7.0	.52	1.44	113	1.28	92	1.15	34	.64	1040
		.50	1.92	117	1.28	86	1.32	32	.74	1020
	pH 4.0	.015	1.61	102	1.19	100	1.10	36	.68	93
		.012	1.65	99	1.22	86	1.13	25	.69	90
	pH 5.0	.023	1.33	109	1.44	91	1.20	39	.79	66
		.015	1.73	106	1.44	99	1.17	25	.80	80
0.0 p.p.m. Fe										
	pH 6.0	.009	1.55	103	1.04	49	1.25	30	.82	64
		.011	1.84	122	1.36	85	1.30	30	.90	67
	pH 7.0	.009	1.74	90	.95	60	1.23	19	.89	69
		.010	1.89	131	1.03	77	1.34	20	.90	54

fact that most of the iron used by the plant comes from the cotyledons when such a small concentration is available through the nutrient solution. The total amount of iron available from the cotyledons would overshadow that obtained from the nutrient environment (**12**). But as the concentration is increased to 1.0 p.p.m. there is a substantial increase in the rate of uptake at pH 4.0. The observed rate of uptake was not proportional to the increase in iron concentration in the nutrient solution. However, the accumulation of iron on the roots increased greatly between 0.1 and 1.0 p.p.m. of iron added to the nutrient solution at pH 4.0 and approaches a proportional relationship.

At pH 7.0 the uptake of iron into the trifoliate leaves is at a uniform and slightly increasing rate in the range of 0.004 to 1.0 p.p.m. of nutrient iron.

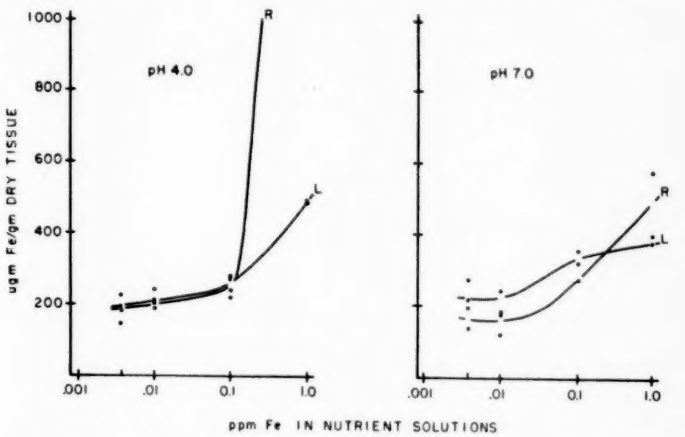

FIG. 4. The effect of iron concentration in a nutrient solution on iron uptake using ferric nitrate as the source of iron. The phosphorus concentration was 0.0005 M. R—roots, L—trifoliate leaves.

This probably is a result of the low availability of iron at high pH values. Likewise, the root accumulation is not heavy and increases only at a low rate within this range of nutrient iron concentrations.

At pH 4.0 the cultures at all concentrations of iron were healthy and green whereas at pH 7.0 only those cultures containing 1.0 p.p.m. of iron did not sustain a chlorosis. Thus, generally speaking, it has been found that 0.1 p.p.m. of iron in a nutrient medium is sufficient iron for normal green growth of the Red Kidney bean plant grown at pH 4.0 and 5.0, but 1.0 p.p.m. of iron sems to be necessary at 6.0 and definitely at 7.0 providing the phosphorus concentration is 0.0005 M or less.

THE EFFECT OF PHOSPHORUS CONCENTRATION ON IRON UPTAKE

Of all the conditions affecting iron uptake that were studied, the effect of phosphorus concentration seemed to be the most complex. Previous

authors (**3, 7**) have investigated the effect of higher concentrations of phosphorus on iron uptake; however, variations from the accepted reaction have been found for lower concentrations of phosphorus.

In figure 5 can be seen the results of three experiments conducted over a range of phosphorus concentrations from 2×10^{-6} to 5×10^{-3} M all at pH 6.0. The plants were grown in a greenhouse where such ambient conditions as sunlight, temperature and humidity were not easily controlled. Because

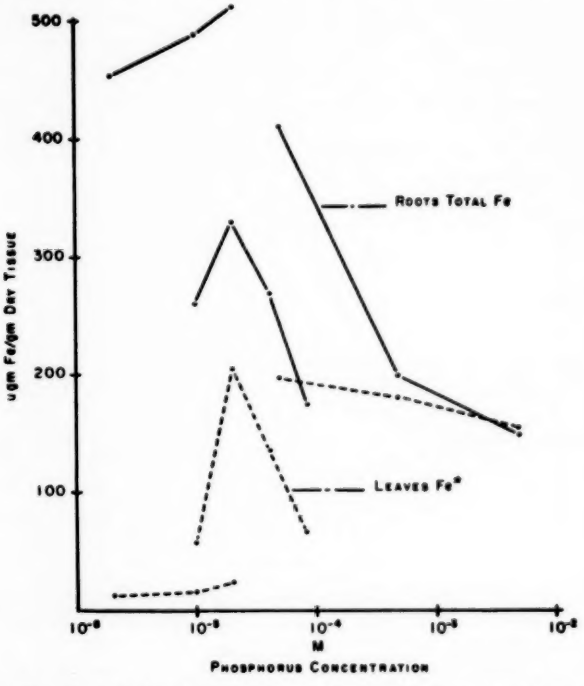

FIG. 5. The effect of phosphorus concentration in a nutrient solution on iron uptake at pH 6.0 using Fe^*Cl_3 as the source of iron during the experimental period. The leaves represent marked iron absorbed during experimental period, whereas the roots represent total iron. Multiply root concentrations by ten. The three groups of data were obtained as separate experiments run at different times.

of this, some variation in absolute values between experiments was obtained since each experiment was presumably conducted under different ambient conditions. These data, however, illustrate one important point, that iron uptake from a nutrient solution was at a maximum when the phosphorus concentration was equimolar with iron; *i.e.*, 2×10^{-5} M. It may also be pointed out that the iron associated with the roots (absorbed and adsorbed iron) was at a maximum at 2×10^{-5} M phosphorus. A second experiment covering the entire range in phosphorus concentration from 5×10^{-6} to

5×10^{-3} M was conducted at a pH of 6.0. Again it was found that uptake into the leaves was at a maximum at the phosphorus concentration nearest to that equimolar with iron; *i.e.*, 5×10^{-5} M. But in contrast to the previous experiments, the accumulation of iron by the roots was greater at the lowest nutrient phosphorus level instead of at the equivalence point between iron and phosphorus (fig. 6). Thus the amount of iron associated with the roots may either increase or decrease as phosphorus concentrations are decreased from those equimolar with iron in the nutrient solution.

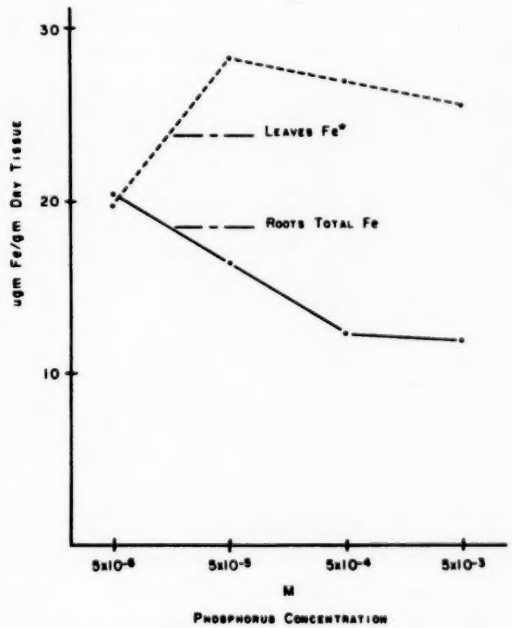

FIG. 6. The effect of phosphorus concentration in a nutrient solution on the uptake of iron at pH 6.0 using 1.0 p.p.m. of Fe^*Cl_3 as the source of iron. The leaves represent marked iron absorbed, whereas the roots represent total iron. Multiply root concentrations by ten.

It appears that when there is more phosphorus than iron in the nutrient medium, the predominant iron precipitate is a ferric phosphate. Analyses have indicated that under these conditions iron and phosphorus appear in the precipitate in an approximate stoichiometric relationship. However, at phosphorus concentrations below those equimolar with iron, the precipitate has been found to be of a ferric oxide type. This latter type of compound may exist in a number of forms, as a hydrosol, a coagulated hydrosol or as crystalline hematite. Obviously, in going from a high phosphorus concentration, 10^{-3} M, to a low phosphorus concentration, 10^{-6} M, one would proceed through various combinations of these two compounds. At the higher

phosphorus concentrations the precipitate would probably be a fine suspension of ferric phosphate, a type of precipitate which apparently is not adsorbed on the roots as well as a gelatinous type. As the phosphorus concentration was decreased, more and more hydrated ferric oxide would be formed which could accumulate on the roots and incorporate that ferric phosphate which was formed with it.

An obvious conditioning factor is a consideration of the isoelectric point described by MATTSON (**6**). If in varying the concentration of phosphorus, the isoelectric point of the particulate material is attained a coagulation and accumulation of the material on the roots will result. Sufficient data is not available to determine the mechanism of this reaction.

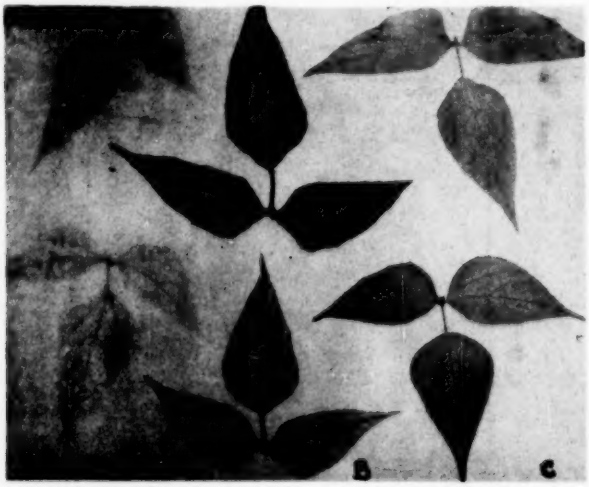

FIG. 7. Autoradiograph of trifoliate leaves of bean plants grown under three different experimental conditions using Fe^{55} as a tracer. Group A—0.001 M P, 1.0 p.p.m. Fe as $Fe^{*}Cl_3$, pH 7.0; group B—0.0001 M P, 1.0 p.p.m. Fe^{*}, pH 7.0; group C—0.0001 M P, 1.0 p.p.m. Fe^{*} and pH 4.0.

The uptake of iron into the trifoliate leaves tends to parallel this accumulation on the roots for high nutrient phosphorus concentrations. This may indicate that that which is adsorbed is ultimately available for absorption and consequent translocation. But as the phosphorus concentration is reduced to less than equimolar with iron, the precipitate becomes predominately a ferric oxide type. GILE and CARRERO (**3**) have shown that ferric oxide is absorbed by plants with difficulty. Thus, less uptake would be expected as the ferric oxide concentration increased. If the precipitate was a crystalline hematite it would account for little accumulation on the roots. Whereas, a gelatinous hydrated ferric oxide type, formed at low nutrient phosphorus levels, causes a heavy deposition of iron on the roots. In either case the uptake into the trifoliate leaves is small.

Phosphorus-induced chlorosis

Considering the preceding work on phosphorus-iron relationships, it is evident that phosphorus exerts a marked influence on the uptake of iron. But not in all cases of iron-deficiency chlorosis does the primary influence seem to be on iron uptake. Rather, the ultimate effect appears to be a blocking of the use of iron after it has been absorbed. Incidents of this nature have been reported by many workers. Upon examination of chlorotic leaves, they were often found to have a higher iron content than normal green leaves. Olsen (7) has described this condition and thought that perhaps it could be merely a precipitation of iron in the veins of the leaf as ferric phosphate. His contentions were upheld experimentally by the use of the Prussian blue test which indicated a heavy deposition of iron in the veins of the chlorotic leaf.

Confirmation of Olsen's work was obtained with Red Kidney bean plants grown in a nutrient solution using radioiron. Figure 7 is an autoradiograph of the trifoliate leaves of bean plants grown under three different experimental conditions. Leaves of group A were taken from plants grown in a nutrient solution containing 0.001 M phosphorus, 1.0 p.p.m. of iron as Fe^*Cl_3, and at pH 7.0. These leaves were fully chlorotic. The phosphorus concentration and pH were high enough to prevent iron in significant quantities from reaching the leaves. This is evidenced by the light exposure obtained on the autoradiograph.

Leaves of group B were taken from plants grown in a nutrient solution containing 0.0001 M phosphorus, 1.0 p.p.m. iron as Fe^*Cl_3, and at pH 7.0. These leaves had a chlorotic mesophyll but were green along the veins, similar to the plants described by Olsen. Upon examination of the autoradiograph of these leaves, it can be seen that there is a heavy deposition of iron in the veins while the mesophyll is relatively free of iron. In this case, the phosphorus concentration of the nutrient solution was low enough to allow absorption of iron at pH 7.0, but the uptake of phosphorus was still great enough to cause precipitation of iron in the veins.

Leaves of group C were taken from plants grown in a nutrient solution containing 0.0001 M phosphorus, 1.0 p.p.m. iron as Fe^*Cl_3, and at pH 4.0. In this case the uptake of iron was rapid and the distribution was uniform enough to produce a normal green leaf. It is obvious that a pH of 4.0 in the nutrient solution in some way inhibits the formation of a ferric phosphate precipitate in the veins of the leaves providing the phosphorus level is maintained below 0.0001 M. At a pH of 4.0 sufficient iron can also be translocated from the cotyledons of the germinating seed to maintain a normal green plant.

This experiment tends to substantiate the supposition that certain types of chlorosis are caused by the immobilization of iron in the veins of the leaf. This type of chlorosis is characterized by a chlorotic mesophyll with green veins. Both the pH and the phosphorus content of the nutrient may play a part in its immobilization.

The distribution of iron

In the preceding experiments, the general distribution of iron has been studied on a gross anatomical basis. It was the purpose of this investigation to determine the distribution of iron within each of the plant parts. To accomplish this, use is made of autoradiographs of whole plants that have been grown with 4 different regimes of iron administration. Plants were

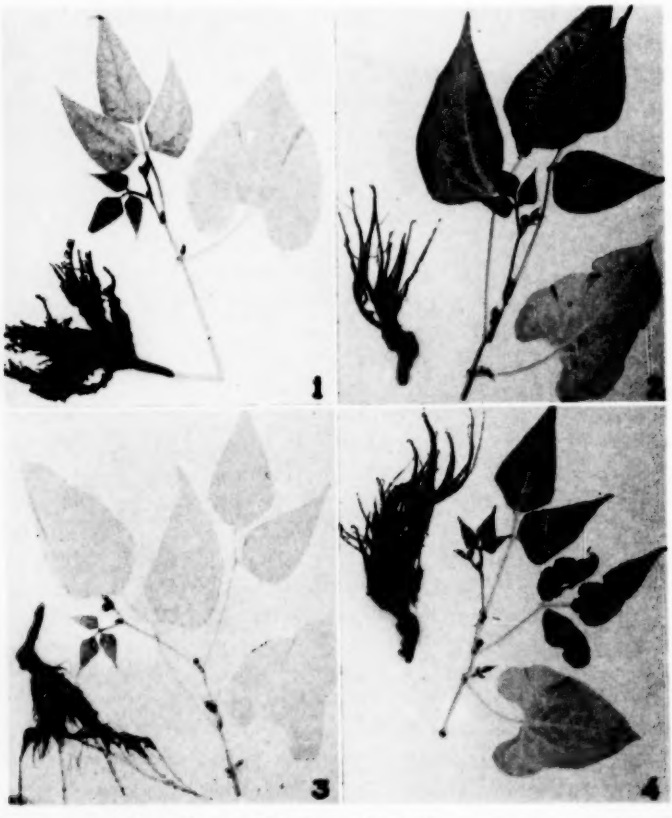

Fig. 8. Autoradiographs of whole bean plants when grown in a nutrient solution containing 1.0 p.p.m. of Fe^*Cl_3. Group 1—10 days with Fe^*; group 2—15 days with Fe^*; group 3—10 days with Fe^* then 5 days with no Fe; group 4—10 days with no Fe then 5 days with Fe^*.

grown in 4 groups with 1.0 p.p.m. of iron as Fe^*Cl_3 available as the source of iron. The phosphorus concentration in each case was 0.0002 M. Group 1 was allowed to grow in a nutrient solution with iron for 10 days at which time it was harvested, 1 plant being prepared for an autoradiograph (fig. 8). The remaining 7 plants were dried for analysis. Group 2 was also allowed to grow with iron available for the 10-day period plus an additional 5 days

as well. The plants were then harvested in the same manner as group 1. The plants in group 3 received the same treatment for the initial 10 days. At this time as much of the adsorbed iron as possible was removed from the roots with an HCl solution of a pH of 3.0. The plants were then placed in a nutrient solution containing no iron for the remaining 5 days. They were then harvested at the end of the 15-day period according to the same plan as group 1. Group 4 was grown without iron for the initial 10-day period causing a rather severe chlorosis of the first trifoliate leaf. It was then placed in a nutrient solution containing iron for the remaining 5 days. The harvest and preparation for analysis were the same as for group 1. Autoradiographs of these plants are shown in figure 8. Complete analyses were performed and concur with the conclusions based on the autoradiographs.

The points of interest gained from this experiment include the following: The distribution of iron in the leaf tissues of healthy plants is surprisingly uniform throughout, with the exception of a slightly higher concentration in the terminal and lateral buds. The rate of uptake of iron by iron-deficient plants is greater than for normal plants at least for an initial period. This is evidenced by plants of group 4 which accumulated as great a concentration of iron during a 5-day period as the normal plants of group 2 accumulated during a 15-day period. The concentration of nutrient iron in the primary leaves of the plants of group 1 appears to be less than in the trifoliate leaves even though the data of table I indicates the primary leaves nearly always have the greater concentration. Thus the initial supply of iron must come from the cotyledons with a later slow uptake from the nutrient solution. The roots are covered by a heavy uniform deposition of iron. If such plants are removed to a solution containing no iron, the iron accumulation is not redeposited on new root growth, but the new leaves formed during this period acquire ample supplies of nutrient iron.

The translocation of iron

The translocation of iron, and the factors affecting it, has long intrigued workers because of its possible close relationship with chlorosis. A number of studies of these factors have been made, particularly the effect of phosphorus. Its effect on the translocation of iron from the roots to the leaves has been most frequently considered, and little attention has been given to its possible effect on the redistribution from the leaf to other parts of the plant. It was the purpose of the following experiments to determine if translocation of iron from the leaf is possible, and if so, to outline some of the factors influencing it.

In this study use is made of the leaf flap injection method of Biddulph (1) whereby iron can be injected into a leaf in such a manner that downward redistribution will occur through the phloem. A number of experimental conditions were applied to the nutrient solutions during the growth of the plants to determine which of those factors known to influence uptake might also influence redistribution of iron.

The iron solution to be injected contained 0.46 mc./ml. of Fe^{55} and 1000

p.p.m. of carrier iron and was injected into a veinlet of the second trifoliate leaf of the bean plant. A 10-minute injection period was allowed. A 24-hour "migration" period was allowed between injection and harvest. Autoradiographs were made of the pressed and dried plants to determine the extent and relative amounts of redistribution that were obtained.

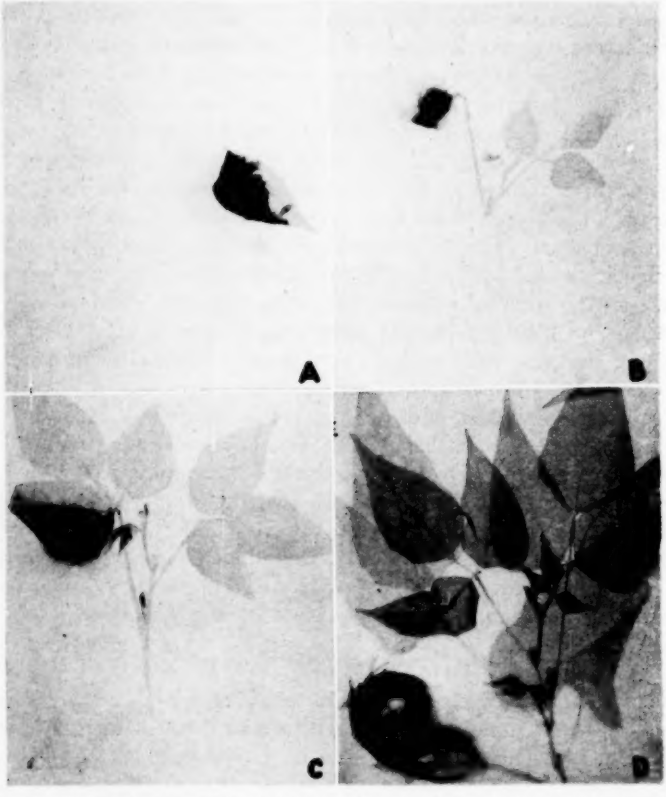

FIG. 9. Autoradiographs of whole bean plants after injection with Fe^*Cl_3 in the second trifoliate leaf when grown in the following nutrient conditions: (A) pH 7.0, P—0.001 M, Fe—1.0 p.p.m.; (B) pH 4.0, P—0.0001 M, Fe—1.0 p.p.m.; (C) pH 7.0, P—0.00001 M, Fe—0.0 p.p.m.; (D) pH 4.0, P—0.000005 M, Fe—0.0 p.p.m. The site of injection is indicated by the intensely exposed area.

An investigation was made of the effect of (1) the pH of the nutrient medium, (2) the iron content of the nutrient medium, and (3) the phosphorus content of the nutrient medium, upon the mobility of injected iron. It was found that each exerted a controlling influence on the amount of iron which was translocated. The least translocation occurred when all three factors were unfavorable to translocation. They were: a high phosphorus concentration, a high pH, and 1 p.p.m. of iron in the nutrient medium. Fig-

ure 9 A represents an autoradiograph of such a plant and it can be seen that practically no iron left the leaf under these conditions. All possible combinations of high and low pH, high and low phosphorus, and high and low iron were tried. The best translocation of iron occurred when the plants were grown in a solution containing very low phosphorus (0.000005 M), no iron, and maintained at pH 4.0. Figure 9 D represents an autoradiograph obtained under those conditions of growth. It is evident that the translocation of iron has been exceptionally rapid and complete. Translocation in the plant represented by figure 9 B has been suppressed by the presence of ample iron in the tissues as a result of having grown the plant with 1 p.p.m. of iron, as well as by an adequate supply of phosphorus (0.0001 M). In the case of 9 C, only the pH of the nutrient, which was at 7.0, has retarded translocation as the plants were grown without iron and at 0.00001 M phosphorus.

It thus is apparent from these experiments that the greatest mobility of injected iron occurred in plants grown with nutrient conditions of low pH and low phosphorus and iron concentrations. The least mobility of injected iron occurred with nutrient conditions of high pH and high phosphorus and iron concentrations. This is verified by figures 9 D and 9 A, respectively. Intermediate conditions of the above factors will yield intermediate degrees of iron mobility.

The primary leaves of the bean plant have long been a curiosity to those who have used this plant for experimental purposes. The primary leaves seem able to retain that iron which they received from the cotyledons in spite of the demand from the trifoliate leaf system which may become fully chlorotic. It is also very difficult to cause a chlorosis of the primary leaves. Situations such as these have caused iron to be labelled an immobile element. However, perhaps it is not that iron is inherently immobile, but rather that conditions such as high phosphorus and high pH, as demonstrated for the trifoliate leaf system, may be operative in preventing the movement of iron from the primary leaves.

To test this hypothesis, the primary leaves of a series of plants grown under a variety of conditions comparable to those listed above, were injected with iron and autoradiographs prepared. It was found that the same conditions which govern the redistribution of iron from the trifoliate leaf system also govern redistribution from the primary leaves as well. This explains why at high pH levels the primary leaves may be green and the trifoliate leaves fully chlorotic under iron deficiency conditions. This also explains why it is almost impossible to produce an iron deficiency chlorosis of the Red Kidney bean plant in a nutrient solution of low pH and low phosphorus concentration. The mobility of iron is sufficiently great under these conditions to allow redistribution from the primary leaves in sufficient quantity to satisfy the needs of the remainder of the plant.

Radioautographs, as used in the above experiments, are perhaps only semiquantitative in scope. However, there exists such a wide difference in

results, ranging from complete immobility of iron from the leaves of plants grown with high iron, high pH and high phosphorus content, to complete mobility of iron in plants grown with low iron, low pH and low phosphorus, that valid conclusions can be drawn.

Summary

1. Red Kidney bean plants were grown in nutrient media with varying quantities of iron and phosphorus and at pH values ranging from 4.0 to 7.0 in an attempt to determine the factors associated with the absorption and translocation of iron in the plant.

2. It has been shown that the iron salt used in the nutrient media exerts a very great effect on the association (absorbed and adsorbed) of iron with the root, but no correlation was found between the actual availability of iron to the aerial parts of the plant and (1) the precipitation rate of iron from nutrient solutions containing various iron salts, and (2) the amount of iron associated with the roots.

3. As the hydrogen ion concentration of the nutrient solution increased, the iron uptake increased. The adsorbed iron on the root also increased.

4. As the iron concentration in the nutrient solutions increased, the iron absorption also increased. This was very marked at pH 4.0 but somewhat less pronounced at pH 7.0.

5. It was found that a high concentration of phosphorus in the nutrient solution retards the uptake of iron. As the phosphorus concentration was increased from that equimolar with iron (at 0.00002 M), both iron uptake into all plant parts and iron adsorption on the root decreased. As the phosphorus concentration was decreased from that equimolar with iron, iron uptake into the aerial parts also decreased, but iron adsorption on the roots was found to either decrease or increase. An explanation for the increased or decreased adsorption on the roots is offered which involves the state of the ferric oxide in the nutrient solution. It is based on the postulation that if the ferric oxide is present in the nutrient solution as a hydrosol or a crystalline precipitate, the adsorbed iron on the root decreases. But, if the ferric oxide is present as a gelatinous coagulated hydrosol, the adsorbed iron increases. The factors responsible for determining the nature of the precipitate were not fully investigated.

6. The rate of redistribution of injected iron within the plant depends on several factors as follows:

The iron concentration of the tissues is the primary factor in determining the mobility of iron. Mobility is greatest when tissue concentrations are lowest and vice versa.

The amount of phosphorus available to the plant is of secondary importance in determining iron mobility. The mobility is increased when phosphorus is low and vice versa.

The pH of the nutrient media is also of secondary importance in determining iron mobility. It is greatest when the pH is low, and least when it is high.

Each factor influencing the mobility of iron exerts its own effect regardless of the presence or absence of other factors, hence mobility is greatest at low iron concentrations, low phosphorus, and low pH. Mobility is least at high iron concentrations, high phosphorus and high pH. Intermediate conditions of the sums of all factors yield intermediate conditions in iron mobility.

7. If the phosphorus concentration of the tissues becomes too high, a chlorosis may occur which is caused by the immobilization of iron in the veins of the leaf. When this type of chlorosis occurs it is usually characterized by a persistent greening along the veins with chlorotic interveinal mesophyll. The term phosphorus induced chlorosis is a realistic designation of this phenomenon.

8. The distribution of iron in healthy bean plants tends to be uniform with slightly greater concentrations in the growing points. The accumulation of iron on the roots from a nutrient solution is heavy and may account for a large percentage of that originally added to the solution.

The Fe^{55} herein employed was obtained from the Isotopes Division of the U.S.A.E.C., Oak Ridge, Tennessee.

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THE ABSORPTION AND TRANSLOCATION OF STRONTIUM BY PLANTS[1]

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Received January 19, 1953

Introduction

The relationship of strontium to plants has been investigated a number of times in the past (**3, 4, 5, 6, 9**) with results which usually indicate that it reacts much as calcium. The uptake has been successfully followed from both a soil and a nutrient solution, but in no case known to the authors has work been done on the factors influencing the uptake of strontium from a controlled nutrient environment. Since strontium has assumed recent importance as a product of nuclear fission, it is of interest to define these factors and to determine the rate of incorporation and the amount of retention by plants.

While the ultimate interest is concerned with the behavior of this element in the soil, it was felt that the many variables involved could be studied most effectively under the more precisely controlled conditions of nutrient culture. Experiments on the effect of nutrient strontium concentration and pH on the uptake, distribution and retention of strontium in various plants and plant parts were therefore conducted. Controlled nutrient culture techniques were employed with Sr^{90} as a tracer. In addition, experiments were performed using one soil typical of the south central Washington area.

Materials and methods

Red Kidney bush beans (*Phaseolus vulgaris* L.) were used as the principal experimental plant. The seed was of pure line stock and was obtained from the California Crop Improvement Association.[2] Four other plants were used: Rutgers tomatoes (*Lycopersicum esculentum*), White Russian wheat (*Triticum vulgare*), Belsford Beardless Barley (*Hordeum vulgare*), and Russian thistle (*Salsola pestifer*). The seed of the latter was collected locally.

[2] University of California, Davis, California.

Plants were cultured in six liters of nutrient solution in enameled refrigerator plans (6 plants per pan) according to a previously outlined technique (**1**). Nutrient solutions were continuously aerated and consisted of the following salts and concentrations: KNO_3—0.0025 M; $Ca(NO_3)_2 \cdot H_2O$—

[1] This work was performed under Atomic Energy Commission Contract no. W-31-109-Eng-52.

0.0025 M; $MgSO_4 \cdot 7\,H_2O$—0.0010 M; and KH_2PO_4—0.0005 M. The micro nutrients were as follows: H_3BO_3—0.5 p.p.m. B; $CuCl_2 \cdot 2\,H_2O$—0.02 p.p.m. Cu; $MnCl_2 \cdot 4\,H_2O$—0.5 p.p.m. Mn; $ZnCl_2$—0.05 p.p.m. Zn; and $Fe(NO_3)_3 \cdot 9\,H_2O$—1.0 p.p.m. Fe. The pH of the medium was adjusted to 6.0, except where varied as an experimental condition, "carrier-free" Sr^{90} (< 0.0001 p.p.m. in final solution) was employed as a tracer at levels of approximately 10^{-4} μc./ml. Except where varied as an experimental condition, inactive strontium as $Sr(NO_3)_2$ was added at a concentration of 1.0 p.p.m. Solutions were maintained at the original volume by daily additions of distilled water, after which the pH was adjusted with 1 *N* NaOH or 1 *N* H_2SO_4. Solutions were changed every 4 days.

Some of the experiments were conducted under greenhouse conditions, others in a special growth chamber which permitted close control over temperature, humidity, and lighting. Details of this growth chamber will be described in a subsequent publication.

The soil culture experiments were carried out by the standard Neubauer seedling technique (**10**), with slight modification. The soil used was an Ephrata fine sandy loam with a pH of 7.6 by the thick paste method. It contained about 10 p.p.m. of strontium, was low in available nutrients, especially nitrogen, and contained very little organic material. Carrier-free Sr^{90} was thoroughly mixed with 100 g. of this soil. It was then dried and placed in a shallow crystallizing dish according to the standard procedure outlined in the reference above. One hundred Belsford beardless barley seeds were planted in the dish and the culture placed in a water bath at 25 ± 1° C under artificial light consisting of a ratio of one daylight fluorescent tube to three white fluorescent tubes at 1000 ± 50 f.c. Above ground parts only were harvested after 18 days.

In the nutrient culture experiments the samples taken from the six plants growing in a single pan were combined for analysis. Results reported are the average of two such composite samples. Roots were analyzed as they came from the solution (omitting any washing which might remove adsorbed and absorbed nutrients). Samples were placed in tared, standard taper Erlenmeyer flasks. The fresh weight was obtained and the material then dried for two days at 70° C. After determining dry weights, the samples were digested with concentrated nitric acid. Ten to 20 ml. of acid was placed in the flask, a 6-inch standard taper chimney fitted to the top of the flask, and the assembly placed on the hot plate at low heat. After the plant material had been reduced to a brown syrupy liquid, the hot plate was turned to medium heat and the flasks allowed to reflux until the contents were clear. Chimneys were then removed and the contents evaporated just to dryness. The residue was dissolved in 5 to 50 ml. of 5 *N* nitric acid, depending on the size of the sample.

One ml. aliquots of these solutions were pipetted onto 1-inch stainless steel plates in duplicate, dried under a heat lamp and counted with a Geiger tube having a 3.0 mg./cm.^{2} window.

Results

The accumulation of strontium by the bean plant

This experiment was designed to determine the rate of accumulation and the total amount of strontium accumulated by the Red Kidney bean plant under controlled environmental conditions over an extended period of time. Plants were grown in the controlled environment chamber. Temperature was maintained at 29 ± 1.5° C during the light hours and 24 ± 1° C during the dark hours with relative humidities of 50 and70% respectively. The temperature of the root environment was maintained constant at 26 ± 0.5° C. Plants were illuminated in 16-hour periods at 1300 ± 100 f.c. from a source containing a 1 : 1 ratio of white and cool white fluorescent tubes.

Plants were grown for a period of 32 days with cultures being harvested every 4 days. At each harvest the nutrient solutions were changed in the remainder of the cultures. In addition to the nutrient salts, 1.0 p.p.m. of strontium as $Sr(NO_3)_2$ was added, containing 9.9×10^5 μc. of Sr^{90} per ml. as a tracer. After the twelfth day strontium was added to only half of the remaining cultures to allow a study to be made of the retention as well as the absorption of this element.

The sampling procedure was designed to follow the progress of strontium accumulation and translocation as precisely as possible. Plants were cut into samples as follows: roots, stems and petioles, primary leaves, axillary leaves, and the original trifoliate leaves along the main stem from 1 (oldest) to 6 (youngest). The 6th trifoliate leaf was fully formed about the 20th day. Flowering began about the 24th day. Data obtained after the 28th day are of doubtful validity since maximum growth in the space available was attained at this time. Although data for only a comparatively few of the tissues are recorded here, the data for all tissues were obtained and agree with the conclusions expressed.

The greatest concentration of strontium was always attained in the oldest leaves with a progressively smaller concentration as the age of the leaf decreased. Thus, the primary leaves attained the highest concentration of strontium of any tissue, 660 μg. Sr/gm. of dry tissue in 24 days. Figure 1 indicates that the first trifoliate leaf had a consistently greater concentration of strontium at any particular time than the sixth or youngest trifoliate leaf. This probably results from a time lag in absorption between the leaves of various ages, since the rates of accumulation for the leaf tissues are approximately equal. As far as the trifoliate leaves were concerned, concentration of strontium within the leaf was not a factor in determining the rate of accumulation of strontium by that leaf.

The first trifoliate leaf of those plants which received no strontium after 12 days retained a high concentration of strontium for the remainder of the 32 days. On the other hand, the sixth trifoliate leaf retained a comparatively low and constant concentration. The obvious conclusion is that there was no significant redistribution of strontium even when a comparatively high concentration gradient existed.

This condition of immobility is confirmed when comparisons of the total strontium present in each tissue are made as in figures 2 and 3. It is apparent that the total strontium in the tissues of those plants which received no strontium after the 12th day remained fairly constant. This indicates that once the strontium has been deposited in the tissues, there is very little redistribution.

The tissues varied markedly in their ability to accumulate strontium. For the plants exposed to strontium for 32 days, the six initial trifoliate leaves rapidly accumulated the greatest amount; most of this total being confined to the older leaves in a progression increasing with age. The axillary trifoliates, being younger than the initial trifoliates, never attained a total strontium content as great as the initial trifoliates; even though the

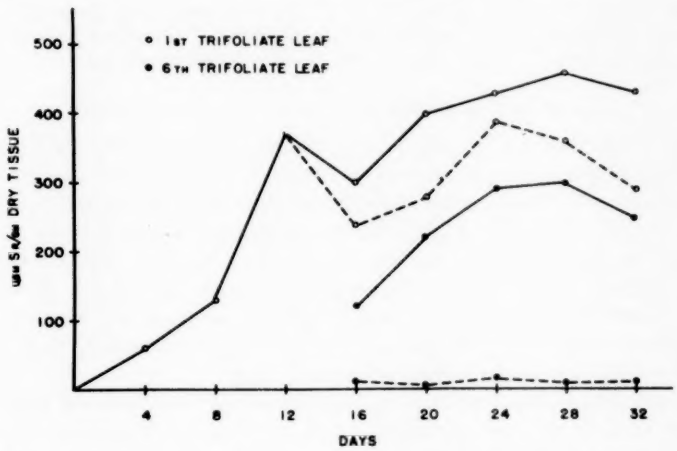

FIG. 1. The concentration of strontium in the first trifoliate leaf (oldest) and sixth trifoliate leaf (youngest) of bean plants grown in a nutrient solution containing 1.0 p.p.m. strontium as $Sr(NO_3)_2$ at pH 6.0. Each point represents the average concentration found in one culture of six plants. The dotted line represents those cultures from which strontium was removed after the 12th day.

dry weight of the axillary leaves was greater. This suggests that the roots are limited in their rate of strontium absorption. And since the total aerial portion was increasing much more rapidly in size than the roots, it follows that the rate of accumulation per aerial unit would have to decrease if strontium distribution were equal. However, both the axillary and initial trifoliate leaves continued to increase in total strontium concentration until general senescence began. The primary leaves, on the other hand, soon reached a point of saturation at about 150 μgm. of strontium; the stems appeared saturated at about 120 μgm. of strontium, even though the stem dry weight continued to increase. The roots continued to increase in strontium content throughout most of the 32-day period. As later evidence will indicate, these saturation values are dependent on the concentration of strontium in the nutrient environment.

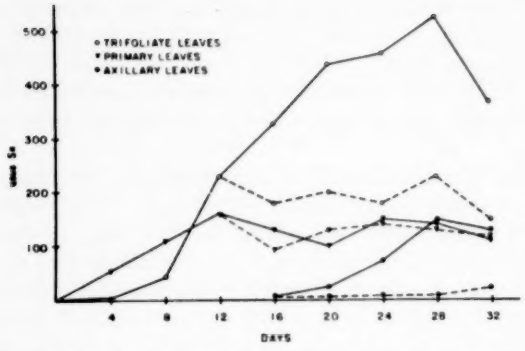

FIG. 2. The total strontium content of the leaf tissue of the bean plant when grown in a solution containing 1.0 p.p.m. of strontium at pH 6.0. The broken line represents those cultures from which the strontium was removed after the 12th day.

THE EFFECT OF CONCENTRATION ON STRONTIUM UPTAKE

Strontium has not usually been considered an element toxic to plant growth; although some authors (**4, 5, 11**) found it to be toxic at very high concentrations. To determine the effect of nutrient strontium concentration on the uptake of the ion, an experiment was conducted over a range of concentrations from 0.0001 to 100 p.p.m., strontium concentration varying by powers of ten. The calcium concentration was maintained at 140 p.p.m. Bean plants were grown for 12 days in nutrient solutions under greenhouse conditions. Strontium was added to the nutrient solution only during the last four days of the experimental period.

It can be seen from figure 4 that the uptake of strontium was generally proportional to the concentration added to the nutrient solution over the entire range of 0.0001 to 100 p.p.m. No toxic symptoms were observed at any concentration. When "carrier-free" Sr^{90} was added to the nutrient solu-

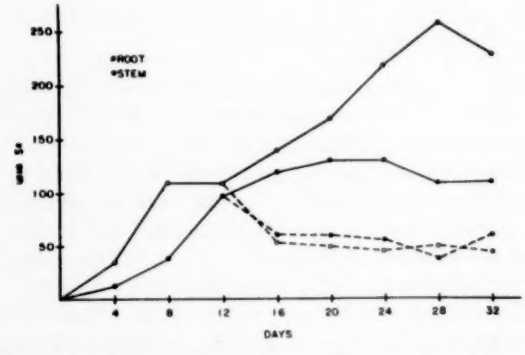

FIG. 3. The total strontium content of root and stem tissue of the bean plant when grown in a solution containing 1.0 p.p.m. of strontium at pH 6.0. The dotted line represents those cultures from which the strontium was removed after the 12th day.

tion, the same proportion was absorbed as when definite amounts of carrier were added.

The amount of strontium associated with the roots was also proportional to the amount added to the nutrient solution, and was always greater than the concentration found in the leaves, suggesting that the strontium associated with the roots was probably composed of adsorbed as well as absorbed strontium. To test this hypothesis, an experiment was performed to determine the difference between strontium accumulation by living roots and by

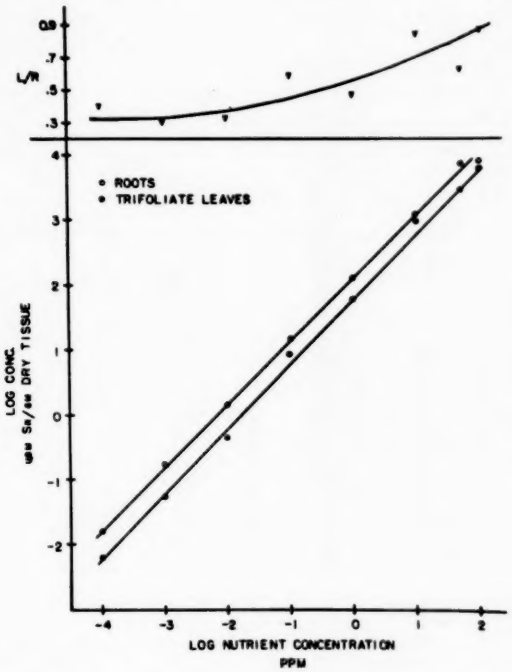

FIG. 4. The uptake of strontium from a nutrient solution containing several concentrations of strontium at pH 6.0. The uptake is expressed as μgm. Sr/gm. of dry tissue. Each point represents the average of duplicate cultures. The L/R ratios are shown in upper graph.

dead roots prepared by drying at 70° C for 24 hours. After four days in a nutrient solution containing 1.0 p.p.m of strontium the dead roots had accumulated an average concentration of 120 μgm. Sr/gm. of dry root tissue which compares with 130 μgm. Sr/gm. accumulated by living roots under the same conditions. These results would seem to indicate that most of the strontium associated with living roots in a nutrient solution is adsorbed strontium, and not truly absorbed as a function of living tissues. JACOBSON and OVERSTREET (7) reached the same conclusion from experiments of shorter duration with excised barley roots. Since the concentration of strontium

associated with the roots is at least 10 times greater than the concentration of strontium in the originally prepared nutrient solution, it is perhaps more realistic to consider this concentration of strontium on the roots as the nutrient concentration of the element, or the concentration to which the absorptive mechanism is actually exposed.

Based on this viewpoint, the ratio of leaf strontium concentration to root strontium concentration (L/R) should be the best measure of strontium

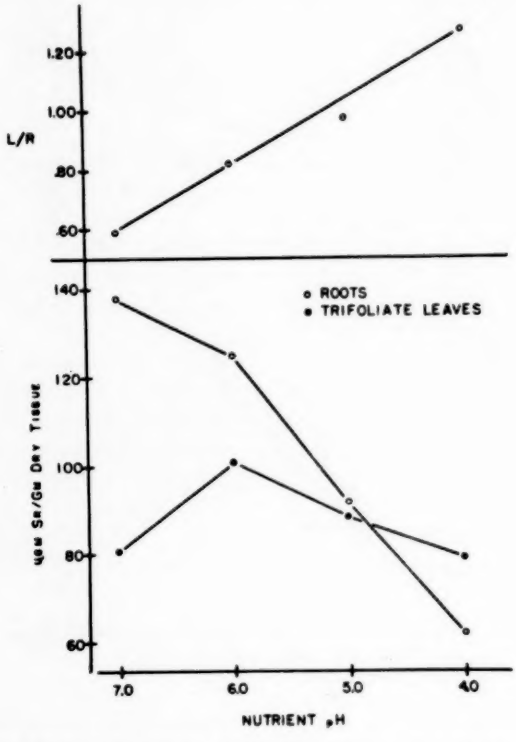

FIG. 5. The uptake of strontium by the bean plant from a nutrient solution containing 1.0 p.p.m. of strontium at various pH levels. The L/R ratios are shown in the upper graph.

uptake efficiency. In figure 4 this ratio is plotted against the concentration of strontium added to the nutrient solution. As the added strontium concentration increases, there is a tendency for L/R to increase, indicating an increased efficiency for strontium absorption.

THE EFFECT OF pH ON STRONTIUM UPTAKE

The effect of pH on strontium uptake was studied in bean plants grown under greenhouse conditions in nutrient solution containing 1.0 p.p.m. stron-

tium. The results are shown in figure 5. The concentration of strontium associated with the roots decreased as the hydrogen-ion concentration increased, due, no doubt, to increased strontium solubility.

The uptake of strontium, as indicated by leaf strontium concentration, is not a smooth function of pH, but exhibits a maximum at pH 6.0. This finding does not appear incongruous if we consider uptake efficiency as measured by the leaf : root ratio. The plot of L/R vs. pH shows that the efficiency of uptake steadily increases as the acidity increases. This increased efficiency at higher acidities is probably due largely to the greater availability of the strontium because of increased solubility. It may also be due in part to enhanced root permeability.

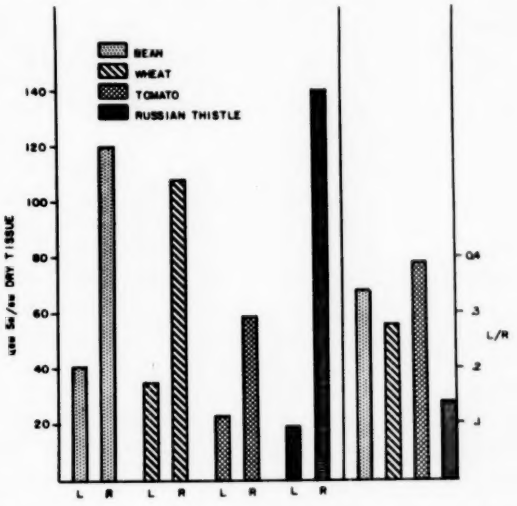

FIG. 6. The uptake of strontium from a nutrient solution at pH 6.0 and 1.0 p.p.m. strontium by four different species. The uptake is represented as μgm. Sr/gm. of dry leaf (L) or root (R) tissue. The L/R ratios are shown on the right.

THE EFFECT OF PLANT SPECIES ON STRONTIUM UPTAKE

Red Kidney beans, tomatoes, wheat and Russian thistle were used to determine the comparative response of these plants to strontium. The plants were grown under greenhouse conditions in a nutrient solution at pH 6.0. Strontium (1.0 p.p.m.) was added to the nutrient solutions during the final 4 days of growth preceding harvesting. Due to the different growth rates of the plants, they were started at different intervals so that all the plants would have an adequate tissue sample for analysis at harvest time. By this means all of the plants were exposed to the experimental conditions during the same 4-day period.

Figure 6 illustrates the differences observed, expressed both as absolute uptakes and as L/R ratios. Greatest variation is seen in the root accumula-

tion, which one might expect in view of the differences in root size and morphology.

As a measure of their absorptive efficiency tomato, bean, and wheat show leaf : root ratios of 0.39, 0.34, 0.28 respectively. Russian thistle is significantly lower with a L/R ratio of only 0.14.

Soil studies

Results of the Neubauer experiments with barley grown in an Ephrata fine sandy loam soil are shown in table I. Absorption of strontium apparently is independent of the Sr^{90} administered to the soil in the range of 0.01

TABLE I

THE UPTAKE OF SR^{90} ABSORBED FROM EPHRATA FINE SANDY LOAM SOIL, CONTAINING ABOUT 10 P.P.M. STRONTIUM AT A pH OF 7.6 BY BARLEY PLANTS USING THE NEUBAUER SEEDLING TECHNIQUE. REPLICATE CULTURES WERE GROWN AS INDICATED.

Dose μc./gm. soil	Dry wt. Tops (gm.)	Conc. factor Dry wt. basis Conc. in tops / Conc. in soil	Per cent of dose absorbed
0.01	1.229	1.3	1.6
	1.269	1.4	1.9
0.05	1.311	.76	1.0
	1.152	1.4	1.7
0.10	1.117	1.6	1.8
	1.083	1.8	1.9
100 p.p.m. Sr added to soil:			
0.01	1.452	1.2	1.8
	1.300	2.0	2.6
	1.282	1.7	2.2
Soil adjusted to pH 4.0:			
0.01	1.245	1.4	1.7
	1.092	1.8	2.0

to 0.10 μc./gm. of soil. The maximum uptake of strontium, under the conditions of this experiment, is only about 1.7% of the total strontium within the root environment. A concentration in the tops is attained about 1.4 times greater than that in the soil as indicated by the average of six replicates. This is substantially in agreement with the work of Jacobson and Overstreet (6) using dwarf pea plants with a 3-month growth period.

Neubauer experiments were also performed with soil to which 100 p.p.m. of strontium was added and with soil adjusted with sulfuric acid to a pH of 4.0. Judging from the previously described nutrient culture results, both of these treatments should have increased the rate of strontium absorption. While the results (table I) do indicate a slightly increased uptake the difference is not significant, considering the limited number of determinations.

Discussion

Most of the emphasis in the study of the absorption of fission products by plants is on being able to estimate the degree of absorption of the element by a plant from its natural environment. This has been done by establishing the maximum uptake obtainable from soils using the Neubauer technique, and supplementing this knowledge with general information on the effect of variables obtained from nutrient culture experiments. The expression of the nutrient culture results in terms applicable to soil conditions is the greatest difficulty in this approach. This difficulty, we believe, has been met by use of the leaf : root ratio as a measure of absorptive efficiency.

In the present nutrient culture experiments with strontium, it was found that massive accumulations of strontium occurred on the roots. This accumulation was in no way connected with metabolic absorption, since it occurred on dead roots to nearly the same extent as on living roots. This adsorption can be associated with several mechanisms most of which would not apply to growth in soils. JACOBSON and OVERSTREET (**6**) have indicated that the ions of this element are simply adsorbed on the root surface, finding that the roots of barley plants could successfully compete with clay particles in removing adsorbed strontium to the roots by exchange. There also has been found in nutrient solutions containing one or more p.p.m. of iron a heavy accumulation of iron on the roots (**8**). The gelatinous ferric oxide matrix could constitute a second site of adsorption. There are sulfates, phosphates, and carbonates in the nutrient solution which undoubtedly precipitate a considerable amount of the strontium present, and this insoluble strontium could well form a heterogeneous accumulation with a ferric oxide on the roots. The concentration of soluble strontium remaining in solution is small in comparison with that adsorbed on the roots, and the significance of this soluble strontium is further reduced because the accumulation of material on the root tends to inhibit direct absorption from the solution (**2**).

It is felt, therefore, that the most significant evaluation of absorption can be obtained by considering the concentration of strontium on the roots as the nutrient strontium concentration affecting absorption. This is accomplished by expressing the data as leaf : root ratios, *i.e.*, the concentration of absorbed strontium found in the aerial portions, divided by the concentration of nutrient strontium associated with the roots. The efficacy of such a procedure is well illustrated in the treatment of the data on the effect of pH on strontium uptake (fig. 5). On a simple concentration basis, the greatest uptake occurs at a pH of 6.0. However, when the data are expressed as leaf : root ratios, the greatest uptake efficiency is seen to occur at a pH of 4.0 with a decreasing linear relationship maintained in the range of pH 4.0 to 7.0. This latter explanation is more consistent with the known effects of pH on solubility and root permeability.

Summary

1. There was found to be no significant redistribution of strontium within the leaf system of the bean plant grown at a pH of 6.0, even though favor-

able concentration gradients existed. The total amount of strontium which a trifoliate leaf will accumulate is proportional to the age of the leaf. However, each tissue appears to have a maximum total amount of strontium which it will accumulate for given nutrient conditions. This maximum accumulation is attained at some characteristic time before senescence, depending on the tissue.

2. It was shown that the absorption of strontium is proportional to the concentration in the nutrient solution up to 100 p.p.m. This indicates that there is no deleterious effect of strontium concentration on the uptake mechanism below 100 p.p.m.

3. The accumulation of strontium on the root decreases as the acidity is increased between pH 7.0 and 4.0. The ratio of leaf strontium concentration to root strontium concentration increases linearly as the pH decreases.

4. It was found that tomato will maintain a concentration of strontium in its leaves, which is 39% of that found on the roots. The percentage for the bean was 34; wheat, 28; and Russian thistle, 14.

5. In Neubauer seedling tests, barley was able to remove to its leaves only about 1.7 per cent. of carrier-free Sr^{90} incorporated in an Ephrata fine sandy loam soil, or to concentrate the strontium in its leaves about 1.4 times over the concentration present in the soil.

The authors are indebted to Miss Josephine Sommers for technical assistance in these studies and to the Biological Services Unit for the radiochemical analysis.

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CONCENTRATION AND TRANSLOCATION OF NITROGEN COMPOUNDS IN THE CORN PLANT (*ZEA MAYS*) DURING GRAIN DEVELOPMENT[1]

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Received May 31, 1951

The purpose of this investigation is twofold: 1. to determine whether the nitrogen in the vegetative parts of the corn plant at pollination is adequate to supply the maturing grain, if not, the contribution of soil nitrogen to the overall nitrogen supply after pollination; 2. to determine to what extent certain nitrogen compounds in the stalks and leaves at pollination contribute to the nitrogen of the maturing grain. The plan was to study corn grown under field conditions on a naturally fertile soil in which there was no deficiency in the nutrient-supplying capacity for the crop. It was also hoped this investigation would disclose new facts about the nitrogen metabolism of the corn plant which might have a bearing on practical aspects of corn protein problems.

There have been many investigations concerned with various phases of nitrogen metabolism of plants. The bulk of the recent work has dealt with the effect of changing environmental and nitrogen nutritional conditions on the composition of the plant (**30, 31, 32, 33, 34, 35**). In view of the many excellent reviews on the subject (**5, 17, 20, 27, 28**) no attempt will be made to review the literature here.

Methods

PLANTING AND SAMPLING

To accomplish the aims set forth in the introduction, two corn hybrids, U.S. 13 and Funk's G-80, were planted in a rich dark-colored soil classified as Drummer clay loam. No commercial fertilizer was applied for this crop, although a good crop of second-year mammoth clover was plowed under.

The hybrids were planted June 1, 1945, and grown at the rate of three plants per hill. To provide for the sampling technique, each hybrid plot was divided into 10 blocks. Only the even-numbered hills were used for samples. At sampling, one from each block was chosen at random. The plants were examined and those showing too much deviation from the average stage of development were discarded. The remaining plants were used as a composite sample, which consisted of the above-ground parts of 14 to 26 plants.

[1] This investigation was supported by a cooperative gift fund from Funk Bros. Seed Company, Bloomington, Illinois, Mr. George M. Moffett, Chairman of Board of Directors of Corn Products Refining Company, and Corn Industries Research Foundation.

[2] Present address: Charles F. Kettering Foundation, Dayton, Ohio.

When U.S. 13 was first sampled on July 20, 50 days after planting, it was still in the vegetative condition. The second sampling was made August 17, 78 days after planting, when pollination was occurring. The following are given in this same order, Aug. 23, 84 days, early milk stage; Aug. 30, 91 days, early dough stage; Sept. 6, 98 days, dough stage; Sept. 20, 112 days, 50% dent; Sept. 27, 119 days, dent; and Oct. 4, 126 days, mature. G-80 was in approximately the same stage of development at corresponding sampling dates.

The plants of the composite sample were dissected into their constituent parts of upper leaves, lower leaves, upper stalk, lower stalk, tassel, shank, husk, silk, and grain. The upper and lower portions of the plant were separated at the point of attachment of the primary ear. The sheath was considered as part of the leaf. Justification for combining the leaf blade and sheath has since been substantiated by the chemical analyses of GIBBS (**9**). The composite plant parts were weighed immediately after separation, then chopped in a Hobart food chopper and representative aliquots taken for moisture determination, oven drying, and drying under vacuum from the frozen state.

SAMPLE PRESERVATION

The material to be oven dried was placed in a forced draft oven at 80° C until dry. The material to be lyophilized was frozen in the field with dry ice, as soon as possible after dissecting and weighing. By freezing the young ears the small developing ovules could be forced intact from the cob, making a clean separation. The frozen material was placed in flasks and kept frozen under vacuum until dry. The apparatus used for drying material while frozen was designed by Earley and DeTurk. Different types of apparatus for drying material while frozen, as well as various uses for the lyophilizing apparatus, have been reported by LINK and co-workers (**14**). It is believed that most nitrogen constituents of lyophilized plant material are little changed from their status in the fresh state. NIGHTINGALE and co-workers (**21**) detected no changes in total N, amide, amino, ammonia or nitrate N content of plant material frozen at – 5° C to – 14° C for 48 hours, and also for 7 days when compared with fresh material.

CHEMICAL ANALYSIS

Total N of the grain was determined according to the KJELDAHL-GUNNING-ARNOLD method (**2**) using copper as a catalyst. Total N of the vegetative parts was determined by RANKER'S method (**24**) to include nitrates. Total protein N was obtained by subtracting the non-protein N of the water-soluble fraction from the total N of the samples. The authors are aware that these values also include the alcohol-soluble N of chlorophyll and certain other compounds which are not protein in nature. However, these are believed to make up a minor portion of the total.

Lyophilized material of U.S. 13 was used for a detailed study of the

water-soluble N. The sample size used for extraction was varied from 3 to 5 gm. depending on its nitrogen content. The enzymes were inactivated by extracting the plant materials at 80° C, as suggested by VICKERY *et al.* (**29**). While the samples were in the 80° C bath, they were stirred constantly. This stirring was followed by centrifugation, decanting, and successive extractions with cool water until a negative test for nitrate was obtained using BRAY's technique (**3**). An aliquot of the supernatant liquid was analyzed immediately. A few drops of monochlorobenzene were added to the remainder of the liquid before it was stored under refrigeration for possible rechecks.

Water-soluble N was determined by the reduced-iron-powder method of PUCHER and co-workers (**22**). Water-insoluble N was obtained by subtracting the water-soluble N.

Proteins were removed from the water extract with sodium tungstate at pH 2.1 (**11, 18, 25**) before the determination of NH_3, amide, and amino N in the water extract. Non-protein N was determined on an aliquot of water extract from which the proteins had been precipitated. A micro adaptation of the reduced-iron-powder method was used for the digestion of the sample (**15, 22**). The sample maintained at 55° C, was aerated into standard acid. Ammonia was then determined by NESSLER's method (**12**) using a Coleman spectrophotometer at 500 mμ. Free ammonia of the protein-free extract was also determined in this same manner.

The method used for the determination of nitrate N in the water extract was based on the work of BURSTROM (**4**), FREAR (**8**), and HARPER (**10**) using phenoldisulfonic acid. Absorption of the resulting solution was determined in the spectrometer at 410 mμ.

The stability of the amide group of glutamine during the removal of free NH_3 N has long been a concern of plant physiologists. However, it has been shown by VICKERY and co-workers (**29**) and by tests at this laboratory that the amide-N glutamine is stable under conditions set forth in the free NH_3 determination.

The method outlined by VICKERY *et al.* (**29**) was used, with minor changes, in the determination of glutamine amide N. The protein-free water extract was adjusted to pH 6.5 with NaOH using a phosphate-borax buffer. After a two-hour period in a boiling water bath the total NH_3 was determined. Glutamine amide N was obtained by subtracting the free NH_3 N value from the total NH_3 obtained in this determination. The procedure of PUCHER and co-workers (**23**) was followed with minor changes. An aliquot of the protein-free water extract was placed in a boiling water bath for 3 hours with no adjustment of pH since this was about 2. The total NH_3 N was removed by aeration and determined. The value previously obtained for free NH_3 N and glutamine amide N was subtracted from the value obtained in this determination and the remainder was considered asparagine amide N.

The method used for the estimation of the free α-amino N was one modified from that of MACFAYDEN (**16**). The value obtained using this method

includes the free amino N of the amides as well as that of the amino acids. A 10-ml. aliquot which had been freed of proteins was transferred to an aeration tube and adjusted to pH 2.5 with a citrate buffer. Ninhydrin was added at the rate of 20 mg. per ml. of solution. The tube was then placed in a boiling water bath for 30 minutes. After cooling, the sample was made alkaline to phenolphthalein with NaOH and NH_3 determined. The value of the free NH_3 N was subtracted from the NH_3 value obtained by this procedure and the remainder was considered an estimate of the free α-amino N.

Discussion of results

Total nitrogen

Data in all tables and figures are based on the average plant of each particular sample. Table I gives the dry weight of the average corn plant at each sampling date. Both hybrids followed very much the same trends of growth. The most rapid rate of dry matter production for the plant minus grain was during the first week following pollination. Sayre (**26**) sampled Ohio K35 every 3 days and reported a maximum rate of dry matter production during the time of silking and tasseling. The loss of plant weight of U.S. 13 and G-80 which followed the seventh sampling was probably due to the natural loss of dry leaves and tassels. This loss is an unavoidable error in field-grown material. The maximum weight of the whole plant

TABLE I

DRY WEIGHT OF PARTS OF THE CORN PLANT WITH ADVANCING MATURITY (VALUES IN GRAMS).

Date sampled	Upper leaves	Lower leaves	Upper stalk	Lower stalk	Shank	Husk	Cob	Total minus grain	Grain	Total
				U.S. 13						
July 20	15.0*	....	9.1†	....	....	....	....	24.1	....	24.1
Aug. 17	35.6	26.6	14.7	60.4	2.5	8.8	....	148.6	7.3‡	155.9
Aug. 23	41.4	37.1	18.2	68.9	5.0	23.8	21.4	215.8	6.1	221.9
Aug. 30	35.9	35.4	15.3	71.0	4.5	22.1	31.8	216.0	25.3	241.3
Sept. 6	42.1	33.5	18.1	62.9	6.5	32.8	40.3	236.2	65.8	302.0
Sept. 20	33.0	28.8	13.9	46.8	6.2	16.9	34.2	179.8	121.8	201.6
Sept. 27	34.6	35.1	14.4	58.4	5.2	21.6	37.6	206.9	151.0	357.9
Oct. 4	30.5	26.5	13.1	51.7	4.2	17.6	35.9	179.5	161.4	340.9
				G-80						
July 21	13.9*	....	8.4†	....	....	....	....	22.3	....	22.3
Aug. 21	40.0	31.3	16.9	55.8	3.8	17.3	....	165.1	14.2‡	179.3
Aug. 27	42.4	31.9	18.1	58.4	4.7	22.2	24.8	202.5	7.3	209.8
Sept. 4	46.6	32.4	20.3	60.3	6.5	25.1	41.6	232.8	43.1	275.9
Sept. 11	41.8	33.8	16.0	54.0	6.4	24.2	38.3	214.5	87.3	301.8
Sept. 18	45.0	27.8	17.5	55.3	6.1	23.2	36.7	211.6	108.5	320.1
Sept. 25	37.0	26.6	16.7	47.2	5.6	17.9	36.1	187.1	146.0	333.1
Oct. 2	29.8	27.2	13.4	46.3	4.6	13.8	31.0	166.1	143.4	309.5
Oct. 9	27.3	24.5	15.9	55.1	5.3	19.6	41.0	188.7	183.9	372.6

*All leaves; †whole stalk; ‡ grain and cob.

minus the grain was reached at approximately the same time by both hybrids, G-80 at 96 days after planting and U.S. 13 at 98 days, which correspond to the early dough and dough stages, respectively. However, the increase in dry weight from the early milk stage to maximum dry weight averaged only 12.7%. Also, the grain of neither hybrid was fully matured by the last sampling period as evidenced by a moisture content above 35%. Since a frost occurred a few days before the last sampling, it is highly improbable that a significant increase in the dry weight of grain per plant occurred after the sampling.

TABLE II

GRAMS OF NITROGEN IN PARTS OF THE CORN PLANT WITH ADVANCING MATURITY (OVEN-DRIED SAMPLES).

Date sampled	Upper leaves	Lower leaves	Upper stalk	Lower stalk	Shank	Husk	Total minus grain	Grain	Total
				U.S. 13					
July 20	0.496*	....	0.293†	....	....	....	0.789	....	0.789
Aug. 17	0.833	0.630	0.184	0.574	0.054	0.139	2.414	0.188‡	2.602
Aug. 23	0.898	0.768	0.169	0.516	0.078	0.250	2.679	0.179	2.858
Aug. 30	0.729	0.733	0.122	0.535	0.055	0.188	2.362	0.660	3.022
Sept. 6	0.846	0.690	0.135	0.429	0.069	0.258	2.427	1.303	3.730
Sept. 20	0.505	0.458	0.092	0.297	0.031	0.103	1.486	1.949	3.435
Sept. 27	0.457	0.463	0.087	0.327	0.037	0.104	1.475	2.418	3.893
Oct. 4	0.381	0.363	0.083	0.334	0.033	0.073	1.267	2.566	3.833
				G-80					
July 21	0.493*	....	0.265†	....	....	....	0.758	....	0.758
Jug. 21	0.956	0.754	0.167	0.523	0.060	0.232	2.692	0.341‡	3.033
Aug. 27	0.882	0.657	0.139	0.432	0.053	0.204	2.367	0.223	2.590
Sept. 4	0.913	0.635	0.135	0.420	0.062	0.195	2.360	0.931	3.291
Sept. 11	0.757	0.619	0.087	0.304	0.052	0.147	1.966	1.528	3.494
Sept. 18	0.778	0.514	0.102	0.311	0.050	0.140	1.895	1.801	3.696
Sept. 25	0.585	0.426	0.079	0.231	0.036	0.088	1.445	2.219	3.664
Oct. 2	0.408	0.384	0.072	0.242	0.035	0.061	1.202	2.223	3.425
Oct. 9	0.330	0.345	0.089	0.366	0.040	0.091	1.261	2.832	4.093

*All leaves; †whole stalk; ‡grain and cob.

The moisture content of the leaves of both hybrids was nearly the same at the second sampling and remained relatively constant until after the frost. The upper leaves always contained a slightly lower percentage of moisture than the lower leaves, but following frost this difference increased until the upper leaves contained only a little more than half as much moisture as the lower leaves. The main conducting parts of the corn plant (stalk, shank, and cob) carried considerably higher percentages of moisture at the end of the season than did the husk, grain and leaves, even though all parts contained about the same percentage of moisture earlier in the season.

A study was made of the gains and losses of nitrogen in parts of the corn plant at various sampling periods. The data from this study are given in table II. Each of the vegetative parts of U.S. 13, except the stalk, contained

the largest quantity of nitrogen on August 23 at the early milk stage of the grain, or 84 days after planting. The upper and lower parts of the stalk contained the largest quantity of nitrogen on August 17, about the middle of the pollination period. The vegetative parts of G-80 showed their peak nitrogen content at a slightly earlier stage of development; that is, mid-pollination, or 82 days after planting. These peaks are in agreement with the ones found by SAYRE (26) in the leaves and stalks of Ohio K35 in 1940. He reported that the greatest nitrogen accumulation occurred near pollination, or about 72 days after planting. Apparently Ohio K35 is a slightly

TABLE III

THE COMPARATIVE CONTRIBUTIONS OF PLANT NITROGEN AND SOIL NITROGEN TO THE DEVELOPING CORN KERNEL.

Periods studied	Periodic increase in grain nitrogen	Grain nitrogen contributed by		Grain nitrogen contributed by	
		Plant	Soil*	Plant	Soil*
	gm.	*gm.*	*gm.*	%	%
		U.S. 13			
Aug. 23–30	0.481	0.317	0.164	65.9	34.1
Aug. 30–Sept. 6	0.643	0.065	0.578	10.1	89.9
Sept. 6–20	0.646	0.941**	0.0	100.0	0.0
Sept. 20–27	0.469	0.011	0.458	2.4	97.6
Sept. 27–Oct. 4	0.170	0.208**	0.0	100.0	0.0
Total	2.409	1.542	1.200	56.2	43.8
		G-80			
Aug. 27–Sept. 4	0.708	0.007	0.701	1.0	99.0
Sept. 4–11	0.597	0.394	0.203	66.0	34.0
Sept. 11–18	0.273	0.071	0.202	26.1	73.9
Sept. 18–25	0.418	0.450**	0.0	100.0	0.0
Sept. 25–Oct. 2	0.004	0.243**	0.0	100.0	0.0
Oct. 2–9	0.609	0.059	0.550	9.7	90.3
Total	2.609	1.224	1.656	42.5	57.5

*Part of the "soil" nitrogen may have come from the corn roots.
**Excess losses possibly due to sampling error or loss of nitrogen to soil.

earlier hybrid than those studied here. The nitrogen contributions of the vegetative parts to the maturing grain can be followed in table II. The upper stalk and husks of both hybrids lost a greater percentage of their nitrogen to the developing grain than any of the other parts of the plant. From a quantitative standpoint the greatest contributions of nitrogen in both hybrids was made by the upper leaves, followed by the lower leaves.

Data presented in table III give the periodic increases of grain nitrogen during maturation. In studying the losses of nitrogen from the vegetative tissues during these periods it was noted that the losses did not account for the increases in the grain nitrogen. It was assumed, therefore, that part of the grain nitrogen must have been absorbed from the soil or the roots, or both. In some instances the losses of nitrogen from the plant parts exceeded

the nitrogen increases in the grain. The reason for this is not definitely known. However, these losses usually occurred during dry weight losses and may indicate sampling errors. The other possibility, though very unlikely (**26**), is a loss of nitrogen from the plant to the roots or soil. Neither supposition can be proved by this study. There was considerable variation in the quantity of nitrogen and in the proportion of plant and soil nitrogen translocated into the grain of U.S. 13 and G-80 during the periods studied. Since both hybrids were planted the same day and sampled at similar stages of development, the differences in grain nitrogen increases for the various periods indicate that the metabolic processes of these hybrids proceeded at different rates. It was not surprising to find that these two corn hybrids translocated nitrogen from the plant into the grain at a faster rate than it was absorbed from the highly fertile soil. This fact had been previously established for the corn plant by EARLEY and DETURK (**6**) and SAYRE (**26**) and for the cotton plant by ARMSTRONG and ALBERT (**1**).

TABLE IV

PERCENTAGE OF TOTAL NITROGEN AND NITROGEN IN THE FRACTIONS OF THE PARTS OF THE CORN PLANT AT DIFFERENT STAGES OF GRAIN DEVELOPMENT (LYOPHILIZED SAMPLES).

Stage of development	Date of sampling	Total N	Water-insol. fraction	Water-sol. fraction	Protein fraction	Water-sol. non-protein fraction
			Upper leaves			
Pollination	Aug. 17	2.33	1.90	0.43	2.07	0.26
Early milk	Aug. 23	1.98	1.65	0.33	1.72	0.26
Dough	Sept. 6	1.77	1.46	0.31	1.59	0.18
Mature	Oct. 4	1.18	0.81	0.38	1.03	0.15
			Lower leaves			
Pollination	Aug. 17	2.36	1.82	0.54	2.02	0.34
Early milk	Aug. 23	1.97	1.60	0.38	1.72	0.25
Dough	Sept. 6	1.98	1.64	0.34	1.76	0.22
Mature	Oct. 4	1.40	0.99	0.41	1.17	0.23
			Upper stalk			
Pollination	Aug. 17	1.32	0.78	0.54	0.91	0.41
Early milk	Aug. 23	0.86	0.49	0.37	0.61	0.25
Dough	Sept. 6	0.69	0.38	0.31	0.42	0.27
Mature	Oct. 4	0.54	0.38	0.16	0.39	0.15
			Lower stalk			
Pollination	Aug. 17	0.83	0.22	0.61	0.42	0.41
Early milk	Aug. 24	0.73	0.27	0.46	0.41	0.32
Dough	Sept. 6	0.62	0.26	0.36	0.32	0.29
Mature	Oct. 4	0.57	0.32	0.25	0.34	0.23
			Grain			
Early milk	Aug. 23	3.38	1.60	1.78	2.01	1.37
Dough	Sept. 6	2.00	1.45	0.55	1.73	0.27
Mature	Oct. 4	1.66	1.44	0.22	1.54	0.12

While it is important to learn precisely why nitrogen and other elements (**26**) decrease in the vegetative parts of plants during grain development, the data in table II reveal the important fact that the corn plant has the capacity to store a large quantity of nitrogen by pollination time and to utilize over half this amount in the development of grain. Therefore, the importance of having an adequate amount of available nitrogen in the soil, especially during the early life of the corn plant when absorption of nutrients is taking place rapidly, is obvious.

Nitrogen fractions

A study of the water-soluble N in the plant parts of U.S. 13 at four stages of grain development was made. Lyophilized samples of stalk, grain and leaves, except the lower leaves at the last sampling, were used for total and water-soluble N determinations. The lower leaves at the last sampling had reached a comparatively dry state in the field and were oven-dried.

The percentages of total N, water-insoluble N, water-soluble N, water-

TABLE V

NITROGEN IN THE FRACTIONS EXPRESSED AS PERCENTAGES OF THE TOTAL NITROGEN OF THE RESPECTIVE PARTS OF THE CORN PLANT AT DIFFERENT STAGES OF GRAIN DEVELOPMENT.

Stage of development	Water-insol. fraction	Water-sol. fraction	Protein fraction	Water-sol. non-prot. fraction	Breakdown of non-protein fraction					
					NO_3 N	NH_3 N	Amide-N		α-amino N	Residual N
							Glut.	Asp.		
Upper leaves										
Pollination	81.5	18.4	88.9	11.1	0.25	0.0	0.87	0.0	0.78	9.23
Early milk	83.3	16.5	86.8	12.9	3.51	0.0	0.86	0.0	1.79	6.82
Dough	82.6	17.2	90.0	10.4	3.21	0.0	0.0	0.84	1.58	4.77
Mature	68.3	31.7	87.2	12.7	1.71	0.0	1.35	0.0	2.06	7.58
Lower leaves										
Pollination	77.1	22.7	85.0	14.4	0.66	0.0	0.75	0.0	0.74	12.30
Early milk	81.2	19.0	87.3	12.8	4.71	0.0	1.04	0.0	2.18	4.87
Dough	82.8	17.2	88.8	12.7	4.09	0.0	0.0	1.64	1.54	4.00
Mature	70.4	29.6	83.6	16.2	0.0	1.62	0.78	0.06	0.46	13.40
Upper stalk										
Pollination	59.1	40.9	68.6	31.2	15.30	0.95	2.17	2.27	1.40	9.16
Early milk	57.3	42.7	71.0	29.0	1.37	0.98	2.16	1.57	5.59	17.30
Dough	54.9	45.0	60.6	39.1	13.20	2.02	0.0	0.05	1.41	22.50
Mature	69.6	30.4	72.3	27.7	7.30	0.99	0.99	0.0	0.0	18.50
Lower stalk										
Pollination	26.6	73.4	50.2	49.7	31.00	1.48	2.70	2.98	1.70	9.80
Early milk	37.8	62.4	56.1	43.7	2.41	0.0	2.82	1.70	6.62	3.02
Dough	42.3	57.5	52.3	47.7	20.40	1.23	8.17	0.0	0.0	17.80
Mature	56.4	43.5	59.9	40.1	28.50	0.87	1.12	0.0	0.0	9.80
Grain										
Early milk	47.3	52.7	58.9	40.5	0.0	2.87	4.56	1.37	1.41	30.50
Dough	72.5	27.5	86.5	13.5	0.0	0.92	2.48	0.0	0.0	10.00
Mature	86.7	13.3	92.8	7.4	0.0	0.0	0.0	0.0	0.0	7.35

soluble non-protein N, and protein N are tabulated in table IV. The percentage of total N decreased from pollination to maturity in all parts studied. This is a reflection of the more rapid rate of carbohydrate synthesis. For the most part the same statement holds true for all classes of nitrogen compounds in all parts of the plant, with the principal exception of the water-insoluble N of the lower stalk.

Table V lists the percentage of the total N of each part that is accounted for by each of the various classes of nitrogen compounds at each stage of development. The water-insoluble N of the upper leaves, lower leaves, and upper stalk, when considered separately, remained fairly uniform throughout the season and averaged 79, 78, and 60% of the total N respectively. In the case of the lower stalk and grain the percentage of water-insoluble N increased with maturity. The water-soluble N of the upper leaves, lower leaves, and upper stalk, when considered separately, also remained fairly uniform during the season and averaged 21, 22, and 40% of the total N

TABLE VI

N FRACTIONS OF PARTS OF THE CORN PLANT AT DIFFERENT STAGES OF GRAIN DEVELOPMENT IN MG.

Stage of development	Total N	Water-insol. fraction	Water-sol. fraction	Protein fraction	Water-sol. non-protein fraction
		Upper leaves			
Pollination	829	676	153	737	92
Early milk	820	683	137	712	108
Dough	745	615	130	669	76
Mature	360	246	114	314	46
		Lower leaves			
Pollination	628	484	144	537	91
Early milk	731	594	137	638	93
Dough	663	549	114	589	74
Mature	371	261	110	310	61
		Upper stalk			
Pollination	194	115	79	133	61
Early milk	157	90	67	111	46
Dough	125	68	57	76	49
Mature	71	49	22	51	20
		Lower stalk			
Pollination	502	134	368	252	250
Early milk	503	189	314	282	221
Dough	388	164	224	203	185
Mature	294	166	128	176	118
		Grain			
Early milk	209	98	111	123	86
Dough	1,316	954	362	1,138	118
Mature	2,679	2,324	355	2,486	193

respectively. The water-soluble N of the lower stalk and grain decreased rather sharply for each successive sampling. In the case of the grain the water-soluble N constituted 52.7% of the total N at the early milk stage and 13.3% at maturity. The proportion of protein N in each vegetative part remained fairly constant throughout the season, while the proportion of protein N in the grain increased with maturity of seed. The average proportion of protein N in each vegetative part was as follows: Upper leaves, 88.2%, lower leaves, 86.2%, upper stalk, 68.1%, and lower stalk, 54.6%. The proportion of water-soluble non-protein N to total N in each vegetative part remained somewhat constant throughout the sampling period. Listed in the increasing percentage of this fraction the order of the plant parts was upper leaves, lower leaves, upper stalk, and lower stalk. The non-protein N of the grain decreased from 40.5% of the total grain nitrogen at the early milk stage to 7.4% at maturity.

The quantity of total, water-insoluble, water-soluble, protein and water-soluble non-protein N per plant part are given in table VI. During the last three stages of grain development the amount of total nitrogen and the four forms of nitrogen decreased in the vegetative parts, with two exceptions, and increased in the grain in all cases. The increases were more than tenfold in the water-insoluble and protein N of the grain and twofold and threefold in the non-protein and water-soluble N respectively. The data of this table indicates an early build-up of the simpler, soluble nitrogen compounds in the corn plant until pollination. After pollination, during the development of the grain, these nitrogen compounds are translocated in some form from the plant to the maturing grain. In the grain there is a build-up of the insoluble protein forms of nitrogen.

A detailed account of the amounts of the water-soluble non-protein fractions is presented in table VII. Nitrate N was present in each part of the corn plant at every stage sampled except that it was never found in the grain and was not present in the lower leaves at maturity. The nitrates showed no indication of uniformly increasing or decreasing with age of the plant. Instead this ion fluctuated widely from one sampling period to another. The lower stalk contained the most nitrates throughout the season, followed by the upper stalk, with the leaves having the least of the vegetative parts. No definite conclusion can be drawn from this work as to the sites of nitrate reduction.

It must be kept in mind that the amount of the various forms of nitrogen measured in the different parts of the corn plant is the amount caught there at the time of sampling. For example, a loss of a certain nitrogen compound from the lower leaves might appear as a gain in the lower stalk as it is being translocated to the grain. Diurnal variations of nitrate N in the cornstalk as observed by KRANTZ (**13**) might partially explain why the nitrate content of the corn plants of this study was so erratic, although all samples were taken the same time of day; that is, about 2 P.M. Nitrogen compounds other than nitrates may be subject to diurnal variations, and therefore, the time

TABLE VII

WATER-SOLUBLE NON-PROTEIN NITROGEN PER PLANT PART AT DIFFERENT STAGES OF GRAIN DEVELOPMENT IN MG.

Stage of development	Non-prot. N	NO_3 N	NO_2 N	Glut. N	Asp. N	α-amino N	Residual N
			Upper leaves				
Pollination	92.2	2.1	0.0	7.2	0.0	6.5	76.5
Early milk	106.0	28.8	0.0	7.0	0.0	14.7	55.9
Dough	77.5	23.9	0.0	0.0	6.27	11.8	35.5
Mature	45.8	6.2	0.0	4.8	0.0	7.4	27.3
			Lower leaves				
Pollination	90.7	4.2	0.0	4.7	0.0	4.6	77.1
Early milk	93.5	34.4	0.0	7.6	0.0	15.9	35.6
Dough	74.7	27.1	0.0	0.0	10.90	10.2	26.5
Mature	60.2	0.0	6.0	1.9	0.23	1.7	49.9
			Upper stalk				
Pollination	60.6	29.7	1.8	4.2	4.4	2.7	17.8
Early milk	45.5	2.2	1.5	3.4	2.5	3.8	27.3
Dough	48.9	16.5	2.5	0.0	0.06	1.8	28.1
Mature	19.6	5.2	0.7	0.7	0.0	0.0	13.1
			Lower stalk				
Pollination	249.0	155.0	7.4	13.5	15.0	8.5	49.2
Early milk	220.0	12.1	0.0	14.2	8.5	33.3	152.0
Dough	185.0	79.3	4.8	31.7	0.0	0.0	69.2
Mature	118.0	83.8	2.6	3.3	0.0	0.0	28.8
			Grain				
Early milk	83.6	0.0	5.9	9.4	2.8	2.9	62.8
Dough	177.0	0.0	12.1	32.6	0.0	0.0	132.0
Mature	197.0	0.0	0.0	0.0	0.0	0.0	197.0

of day of sampling corn for a chemical study should be about the same throughout the sampling period.

No NH_3 N was found in the upper leaves and none appeared in the lower leaves until maturity when 10% of the water-soluble non-protein N was in the form of NH_3. The presence of NH_3 in the lower leaves at maturity was probably due to proteolysis. The proportion of NH_3 in the upper and lower stalk remained fairly constant throughout the sampling period except for the lower stalk at the early milk stage. No explanation can be offered for the absence of NH_3 from the lower stalk at this particular stage of grain development. The NH_3 data indicate that nitrates are reduced in both leaves and stalk of the corn plant but that the most rapid fixation of NH_3 into organic nitrogen compounds takes place in the leaves. The absence of NH_3 in the corn leaves in the presence of nitrates is the basis for the belief that the slow rate of nitrate reduction may have been partly responsible for the decrease in nitrogen in vegetative parts from pollination to maturity. It is also the basis for the supposition that the corn plant might benefit in

yield of grain, and particularly in the protein content of the grain if it were sprayed with a solution of urea or ammonium salts several times from pollination to maturity. Ammonia made up about 7% of the water-soluble non-protein N in the grain at the early milk and dough stages. No NH_3 N was found in the mature grain in this study, although its presence in mature corn grain has been reported by ZELENY (**36**).

The amides varied considerably in the plant parts from pollination to maturity. This may have been due to the normal metabolism of the amides (**5, 19**). Only in the lower stalk was glutamine-amide N found at all stages of grain development. Asparagine was absent from the parts of the corn plant more often than glutamine, but only in the mature grain were both absent from the same part of the plant at the same time. Glutamine and asparagine have been reported in the developing seed of other plants (**7, 35**).

The free α-amino N in the leaves increased from the pollination stage and maintained a relatively high level during the early milk and dough stage samplings, and dropped off rapidly toward maturity. Small amounts were found in the upper stalk at the first three samplings. There was a big surge from the first to the second stages of development in the lower stalk and a complete disappearance following this period. This form of nitrogen was found in the grain only in the early milk samples.

The residual nitrogen, that nitrogen of the water-soluble, non-protein fraction which was not identified as being in a specific form, tended to decrease in the vegetative parts and increase in the grain, table VII. The compounds of this group are believed to be intermediate in complexity between the non-protein moiety studied and the proteins. Therefore it appears that this fraction followed an expected trend by decreasing in the plant and increasing in the grain as it approached maturity.

From table III it was learned that approximately 42.5% of the nitrogen was contributed by the above-ground parts of the plant and that the remainder came from the roots and soil. The nitrogen contributions of each plant part and the various forms of nitrogen cannot be stated with certainty from this study. Loss of the compounds and of total nitrogen can only be considered indications of conversion or translocation.

If one considers the value of the highest accumulation of the nitrogen forms in tables VI and VII and subtracts the lowest value determined for that nitrogen form, the loss or decrease of the compound is the result. By using this method of calculation it can be learned that the leaves lost a larger percentage of water-insoluble N than water-soluble N.; that the upper stalk lost about an equal percentage of these two forms; and that the lower stalk lost much less water-insoluble than water-soluble N. Protein N constituted about 90% of the total N lost from the leaves from pollination to maturity of grain. Also, the greater part of the nitrogen loss of the upper stalk was protein N, whereas that of the lower stalk was non-protein N. The upper and lower leaves contributed an average of 104 mg. of non-protein N to the grain. The nitrate and residual nitrogen made up 28% and 48% respec-

tively, of this amount, with amide and α-amino N each constituting about 12% of this amount. The upper stalk contributed the smallest quantity and the lower stalk the largest quantity of non-protein N to the grain. Of the non-protein N contributed to the grain by the upper and lower stalks, nitrate and residual N made up 44% and 30% respectively. The remaining non-protein N contributed to the grain by the stalk was made up of α-amino, amide, and NH_3 N in decreasing order.

Summary and conclusions

In order to determine the sources of nitrogen for corn grain, a study was made of the chemical composition of various parts of the plant from pollination to maturity. Two corn hybrids, U.S. 13 and Funk's G-80, were used in the total nitrogen study, while the investigation of the water-soluble N was confined to upper and lower leaves, upper and lower stalk and grain of U.S. 13. The plant was divided into the stalk above and below the point of attachment of the shank, upper and lower leaves, grain, shank, and husks. The total, water-insoluble, water-soluble, protein, non-protein, nitrate, ammonia, glutamine amide, asparagine amide, free α-amino, and residual N were measured in the various plant parts. As a result of these studies the following conclusions are believed to be justified.

1. As a general rule per cent. total N decreased in all vegetative parts of the corn plant from pollination to maturity.

2. Several possibilities are suggested for the decline of nitrogen in the vegetative parts of the corn plant during grain development.

3. The grain produced by an average plant of U.S. 13 contained 2.57 gm. of nitrogen at maturity. This amount of nitrogen was equal to 67% of the nitrogen found in the whole plant, including the grain, at maturity. The grain contained a little more than twice as much nitrogen at maturity as did the vegetative parts.

The grain of G-80 contained 2.83 gm. of nitrogen per plant at maturity. The nitrogen of the mature grain made up about 69% of the nitrogen found in the whole plant, including the grain. The grain of G-80 contained about 2.2 times as much nitrogen at maturity as the rest of the plant.

4. Just after pollination there was enough nitrogen in the corn plant to completely supply the maturing grain, if it could have been translocated.

5. The vegetative parts of an average plant of these two hybrids lost 1.61 gm. of nitrogen to the grain. This amount of nitrogen represented an average loss of 57% and constituted approximately 60% of the total grain nitrogen at maturity. Therefore, about 40% of the total grain nitrogen was contributed by the soil and roots after the maximum accumulation of nitrogen in the plant parts had taken place, shortly after pollination.

6. Of the total N contributed by the plant parts to the grain, 60% came from the leaves, 26% from the stalk, 12% from the husk, and 2% from the shank.

7. About 90% of the total N lost from the corn leaves from pollination to maturity was protein N. The greater part of the nitrogen loss of the

upper stalk was also protein N, whereas the lower stalk lost a larger proportion of non-protein N.

8. The study of water-soluble N compounds of U.S. 13 was made in an attempt to learn what forms of nitrogen were furnished from the hydrolyzed proteins and from the incoming nitrogen to the grain for protein synthesis. The residual N showed the greatest loss from the upper and lower leaves, followed by nitrate N. The nitrate N showed the greatest loss from the upper and lower stalks, followed by the residual N. In both the leaves and stalk the contributions of the other forms of water-soluble N to the grain were as follows: α-amino, asparagine, glutamine, and NH_3 N.

9. Plant proteins and the N compounds more complex than amino acids contributed the major portion of nitrogen to the corn grain. Smaller, but nevertheless important, donations of nitrogen to the developing grain were made by the simpler compounds, including nitrate, ammonia, amide, and α-amino N.

10. No final conclusions can be drawn from this investigation as to the chemical route followed by nitrate N previous to its deposition in the grain as organic N. The evidence favors the view that nitrates were converted into protein or protein-like substances in the plant and then translocated to the grain.

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INHIBITION OF AUXIN EFFECTS BY 2,4,6-TRICHLORO-PHENOXYACETIC ACID[1]

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Received June 12, 1952

In a study of the relationship of structure of 2,4-dichlorophenoxyacetic acid (2,4-D) analogs to herbicidal activity, plants treated with 2,4,6-trichlorophenoxyacetic acid (2,4,6-T) did not develop typical epinastic and swelling responses when exposed afterward to vapors of 2,4-D. Further work was carried on with other growth-regulators to determine whether this antagonism was general for auxins or specific for 2,4-D. The growth behavior of treated plants was investigated also to determine if the plant responses to natural hormones could be prevented by 2,4,6-T.

Several auxin antagonists have been reported. Phenylbutyric acid was antagonistic to the stimulating action of indoleacetic acid (IAA) when the two chemicals were applied simultaneously to *Avena* sections (**7**). If the phenylbutyric acid was applied prior to the IAA, the stimulatory action of the IAA was enhanced (**10**). Auxin stimulation of *Avena* sections was inhibited by 2,4-dichloroanisole (**2**). Trans-cinnamic acid antagonized the stimulatory action of 2,4-D, naphthaleneacetic acid (NAA), cis-cinnamic acid, and IAA on the elongation of pea stem sections (**9**). The inhibiting effect of high concentrations of NAA, 2,4-D, and IAA on flax root growth was prevented by 1-naphthol and 2-(1-naphthyl-methylsulfide)-propionic acid (**1**). Maleic hydrazide inhibited the response of pea stems to IAA and NAA (**4**). Naphthyl-phthalamic acid inhibited the phototropic response of peas (**6**).

The effect of 2,4,6-T on plants has been described by several investigators (**3, 5, 8**). It has been found to have no effect on cell elongation or proliferation in tomatoes and snapbeans, but did cause a formative response on foliage, and inhibited root elongation of seedlings. Uupublished work by Mr. J. E. Fisher in this laboratory indicates that 2,4,6-T produces an auxin type of response in soybeans.

Experimental

Chemicals were made up in water with one drop Emulphor-El per 0.100 gm. of chemical as a wetting agent. The inhibitor was used at 100 and 1000 p.p.m.; growth-regulators at 10, 31 and 1000 p.p.m. Bonny Best tomato plants were grown in 2¼-inch clay pots. Plants were used when 7

[1] Journal paper no. J-2080 of the Iowa Agricultural Experiment Station. Project 1139. Additional details of the work are contained in a thesis deposited in the Iowa State College Library.

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to 8 inches tall and growing vigorously. The top 4 to 5 inches of the plant was dipped into solutions of 2,4,6-T. Twenty-four hours later, untreated plants and plants treated with 2,4,6-T were dipped into solutions of growth regulators. A minimum of three plants was used for each treatment.

Epinastic and stem swelling responses of tomato plants dipped in the chemical solutions are shown in tables I and II. Complete inhibition of epinastic response was obtained only at the lower concentrations of growth regulators. One hundred times more inhibitor than growth-regulator was required to give complete inhibition of the epinastic response to 2,4-D.

TABLE I

INHIBITION OF EPINASTIC RESPONSE OF TOMATO PLANTS BY PRIOR APPLICATIONS OF 2,4,6-T.*

Growth regulator	Conc., p.p.m.	Epinastic response**		
		Concentration of 2,4,6-T, p.p.m.		
		0	100	1000
IAA	10	2.0	1.0	0.0
"	31	2.0	1.0	0.3
"	100	3.0	2.0	1.7
"	1000	3.0	3.0	2.0
NAA	10	3.0	0.7	0.0
"	31	4.0	2.0	0.0
"	100	4.0	3.0	1.0
"	1000	4.0	4.0	1.0
BOA	10	0.0	0.0	0.0
"	31	3.0	1.0	0.0
"	100	4.0	3.0	2.0
"	1000	4.0	4.0	4.0
2,4-D	10	3.0	0.7	0.0
"	31	4.0	3.0	1.0
"	100	4.0	3.0	2.0
"	1000	3.0	3.0	3.0
None	....	0.0	0.0	0.0

*Inhibitor applied 1 day before growth regulators. Data taken 20 hours after application of growth regulator.

**Graded on a scale of 0, no response, to 4, maximum response, cf. figure 1a.

For IAA, NAA, and 2-benzothiazolyloxyacetic acid (BOA), 30 times more inhibitor was required. Partial inhibition was obtained with smaller quantities. No inhibition was obtained when the ratio of IAA, NAA, and BOA to inhibitor was 10 to 1.

Stem swelling (table II) was inhibited to approximately the same extent as epinasty. Partial inhibition of swelling caused by IAA and NAA persisted at a 1 to 10 ratio of inhibitor to growth-regulator. Inhibition was completely reversed by BOA and 2,4-D at the higher concentration levels. The highest concentration of 2,4-D and BOA killed the tomato plants in about two weeks. Death of the plants was not prevented by 2,4,6-T, nor did the inhibitor alter the rate at which the plants died.

The rate of growth of the tomato plants affected the degree of response

produced by the growth-regulators. In a test with plants making a slower growth, but otherwise similar to the one for which data are presented, 2,4-D at 10 p.p.m. gave a slight response (rating of 1) and the effect was completely inhibited by 100 p.p.m. of 2,4,6-T.

The antagonistic effect was also demonstrated with other treatment methods. In one test 2,4,6-T, at a concentration of 10% in lanolin applied to tomato stems, inhibited the epinastic effect of later applications of 1% of NAA, IAA, and 2,4-D in lanolin pastes. Curvature of pea stems by 2,4-D was inhibited by a prior immersion of the stems in a 2,4,6-T solution. Ten p.p.m. of 2,4,6-T prevented any curvature by 0.1 p.p.m. of IAA.

TABLE II

INHIBITION OF CELL PROLIFERATION RESPONSE OF TOMATO PLANTS BY PRIOR APPLICATIONS OF 2,4,6-T.*

Growth regulant	Conc., p.p.m.	Cell proliferation response**		
		Concentration of 2,4,6-T, p.p.m.		
		0	100	1000
IAA	10	1.0	0.0	0.0
"	31	1.0	0.3	0.0
"	100	1.0	0.7	0.3
"	1000	2.0	1.0	1.0
NAA	10	2.0	0.0	0.0
"	31	3.0	0.3	0.0
"	100	3.0	1.7	0.0
"	1000	4.0	3.0	1.0
BOA	10	0.0	0.0	0.0
"	31	2.3	1.7	0.3
"	100	4.0	3.3	2.0
"	1000	4.0***	4.0***	3.7***
2,4-D	10	3.0	1.3	1.0
"	31	4.0	4.0	3.0
"	100	4.0	4.0	3.0
"	1000	3.0***	4.0***	1.7***
None	....	0.0	0.0	0.0

*Inhibitor was applied 1 day before the growth regulators; data taken 1 week later.

**Graded on a scale of 0–4 for zero to maximum response.

***All plants dead 2 weeks after treatment.

The time of application of the auxin with respect to the anti-auxin was studied. BOA was applied 2 and 4 hours before, at the same time, and 4 and 24 hours after the 2,4,6-T. Inhibition of the effects of BOA occurred at all of the time intervals. There was a slight reduction in the inhibitory effect when BOA was applied before 2,4,6-T. With simultaneous application of the two chemicals, the inhibition was as great as when the auxin was applied after the anti-auxin. Simultaneous applications of 2,4-D and 2,4,6-T resulted in complete inhibition of the effects of 10 p.p.m. of 2,4-D by 1000 p.p.m. of 2,4,6-T, and nearly complete inhibition by 100 p.p.m. of 2,4,6-T (fig. 1 a).

Since 2,4,6-T inhibited the effect of externally applied auxins, it was of interest to determine whether this chemical would inhibit plant responses controlled by native auxins, such as terminal growth, axillary bud growth, geotropic response of plant stems, orientation of leaves, and rooting of cuttings. Tomato plants treated with 2,4,6-T were placed horizontally (tipped) to determine gravitational responses. Observations were made on treated tomato plants for effects on other responses attributed to auxins, such as axillary and terminal bud growth, and orientation of leaves to sunlight. Plants were treated by dipping into 2,4,6-T solutions.

FIG. 1. Inhibition of auxin effects by 2,4,6-T. (a) Inhibition by 2,4,6-T of the effects of 2,4-D on tomato plants. Treatments L to R, Check, 2,4-D 10 p.p.m., 2,4-D 10 p.p.m. plus 2,4,6-T 100 p.p.m., 2,4-D 10 p.p.m. plus 2,4,6-T 1000 p.p.m. Photographed two days after treatment. (b) Inhibition by 2,4,6-T of gravitational response of tomatoes. Treatments L to R, Check, 2,4,6-T 100 p.p.m., 2,4,6-T 1000 p.p.m.

Tomato plants treated with 1000 p.p.m. of 2,4,6-T and then tipped 90 degrees from the vertical did not respond to gravity to the same extent as untreated plants. The upward curvature was usually considerably less than 90 degrees, and the length of the curved portion of the stem was usually much less than in untreated controls (fig. 1 b). The inhibition of the gravitational response in plants treated with 1000 p.p.m. 2,4,6-T varied all the way from complete inhibition to none. Data for one experiment are recorded in table III. One of the five treated plants failed completely to respond to gravity. The others showed varying degrees of response.

Various factors were studied in an attempt to discover the source of

variation, including the concentration of 2,4,6-T, the time interval between treating and tipping, and the size of the plants. When the concentration of 2,4,6-T was increased to 10,000 p.p.m., the inhibition of the gravitational response was complete. These plants showed foliage injury after 24 hours. A concentration of 100 p.p.m. gave some inhibition. The time interval between treatment with 2,4,6-T and tipping of the plant was varied. Plants were tipped at intervals up to two weeks after treatment. Inhibition of gravitational response was obtained at all of these times. The height of the plant had no effect on the inhibition of the gravitational response. Some plants treated with 1000 p.p.m. 2,4,6-T were not able to orient their leaves so as to get maximum exposure to light. These plants gave only a weak response to the gravitational effect.

TABLE III

INHIBITION OF GEOTROPIC RESPONSE OF TOMATO STEMS BY 2,4,6-T AT 1000 P.P.M.*

Treatment	Height plant	Length of curved portion	Curvature from horizontal degrees
	cm.	*cm.*	
Control	21	11	80
Control	21	10	90
Control	24	16	85
Control	21	13	90
Control	23	10	90
2,4,6-T	24	13	40
2,4,6-T	24	0	0
2,4,6-T	21	8	60
2,4,6-T	20	10	50
2,4,6-T	21	9	90

*Plants were tipped immediately after treatment. Control plants were dipped in tap water.

Coleus cuttings dipped in 100 p.p.m. of 2,4,6-T for 3 hours and then planted in sand failed to root as well as control cuttings. Both the number and length of roots on treated cuttings were less than on untreated cuttings. Roots were located at the basal end of treated cuttings, whereas the control cuttings had roots scattered over the submerged portion of the stem.

Stimulation of axillary bud growth was not observed on any of the treated tomato plants (approximately 200). In one experiment plants were decapitated to induce axillary bud growth. A toxic concentration of 10,000 p.p.m. of 2,4,6-T completely inhibited axillary bud growth, whereas axillary buds on plants treated with 1000 and 100 p.p.m. 2,4,6-T grew at the same rate as those on decapitated control plants. In another experiment (12 treated plants) in which inhibition of gravitational response was complete there was no stimulation of axillary bud growth by 2,4,6-T at 1000 p.p.m. These results suggest that the effect of auxin in inhibiting axillary growth does not involve the same mechanisms as its growth stimulating actions.

Terminal growth was decreased slightly by 2,4,6-T at concentrations of 1000 and 100 p.p.m. In one experiment tomato plants were selected for uniform height and divided into three groups with 27 plants in each group. These groups were given the following treatments: Control, 100 and 1000 p.p.m. of 2,4,6-T. At the end of two weeks growth, the plants had the following average heights: Control, 23.6 cm.; 2,4,6-T at 100 p.p.m., 19.2 cm.; 2,4,6-T at 1000 p.p.m., 18.9 cm.

Discussion and summary

The inhibition of the effects of such structurally diverse compounds as 2,4-D, NAA, IAA, and BOA, by 2,4,6-T suggests that all of these growth-regulators affect similar processes in plants. This inhibition was reversible with large quantities of IAA, NAA, and BOA, indicating the possibility of a competitive antagonism. The results are similar to those obtained by VAN OVERBEEK with cinnamic acid (**9**). Auxin-controlled responses of plants inhibited by 2,4,6-T include epinasty, swelling of stems, negative geotropism of stems, orientation of leaves to light, and rooting of cuttings.

Inhibition of axillary bud growth in plants has been attributed to the effect of a hormone from the terminal bud. Auxins such as IAA applied to the cut end of a decapitated plant have the effect of reducing axillary bud growth. Although 2,4,6-T inhibited other auxin controlled responses, it did not reverse the influence of a terminal bud upon lateral bud growth in these tests.

The antagonism of 2,4,6-T to 2,4-D was first observed while the author was a member of the staff of the Agricultural Chemical Research Laboratory, Naugatuck Chemical Division of the U. S. Rubber Company, Bethany, Connecticut. The work reported here was done at Iowa State College. Appreciation is due to the management of the U. S. Rubber Co. for the generous supply of chemicals used in this later work, and to Drs. W. E. Loomis and S. W. Fox for their helpful suggestions and criticisms.

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STUDIES ON THE CHEMISTRY OF THE LIVING BARK OF THE BLACK LOCUST TREE IN RELATION TO FROST HARDINESS. VI. AMYLASE AND PHOSPHORYLASE SYSTEMS OF THE BARK TISSUES [1]

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Received August 25, 1952

While studying the relation of the chemical composition of the living bark of the black locust tree (*Robinia pseudoacacia*) to its frost resistance, SIMINOVITCH *et al.* (**4, 26, 27**) have observed marked seasonal fluctuations in the starch and sucrose contents of this tissue. The analytical data were suggestive of the existence of a temperature-sensitive starch-sucrose interconversion process, low temperature in late fall promoting a disappearance of starch and warm temperature of early spring promoting the production of starch with a proportionate and simultaneous disappearance of sucrose. Because of the pronounced seasonal changes in the nature of the carbohydrate reserves in the black locust tree bark it was of interest to study enzyme systems which might be involved. Further interest was attached to such a study because little information concerning the enzyme systems present in the living bark of trees is available. The presence of amylase and phosphorylase systems in the bark tissue of the black locust tree is established and characterization studies on these systems are presented here.

The enzymatic degradation of polysaccharides of the starch-glycogen type may be hydrolytic, as catalyzed by amylases, or phosphorolytic, as catalyzed by phosphorylase. The mode of action of the amylases has been the subject of many studies and of several reviews (**3, 10, 22, 25**). Two types of amylase, α and β, are recognized. The α-amylases degrade starch preferentially by hydrolyzing glycosidic linkages far removed from the terminal units. With prolonged incubations, however, α-amylase causes considerable conversion of starch to maltose together with the liberation of a small amount of glucose. β-Amylase degrades starch by hydrolytic cleavage of successive maltose units progressing from the non-reducing ends of the polysaccharide chains. This action is stopped at the branching points of the amylopectin component of starch, but the degradation of the linear component approaches completion. The depolymerizing action of the amyl-

[1] Paper no. 2884, Scientific Journal Series, Minnesota Agr. Exp. Sta. Contents of this paper constitute a part of a thesis submitted by M. H. Ewart to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the Degree of Doctor of Philosophy, March, 1951.

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ases on starch is apparently not reversible, *i.e.*, the equilibrium lies far toward hydrolysis, so synthesis is not observed with these enzymes.

The phosphorolytic cleavage of starch or glycogen involves consumption of one molecule of inorganic phosphate for each glucosidic bond which is broken. CORI and co-workers (**5**) showed that the first product of phosphorolysis of glycogen in animal tissues is glucose-1-phosphate. This reaction is reversible (**6, 7**) and the phosphorylase enzyme catalyzes also the synthesis of polysaccharide from glucose-1-phosphate. The occurrence of phosphorylase in plant tissues was first reported by HANES (**11, 12**) who found it in peas and potatoes. Subsequently the enzyme has been found in many other plant and animal tissues. The reactions catalyzed by the plant and animal phosphorylases are similar. The synthesis of polysaccharide occurs by the endwise apposition of successive glucose units from glucose-1-phosphate to the non-reducing ends of pre-existent starch or glycogen molecules (**15**) which serve as starters. HANES (**12**) and CORI *et al.* (**8, 9**) showed that at a given pH the phosphorylase reaction approaches the same equilibrium ratio of inorganic phosphate to glucose-1-phosphate from either direction. This ratio is independent of the concentration of starch or glycogen but it is pH dependent, varying from about 10.8 at pH 5.0 to 3.1 at pH 7.0 (**12**). HANES and MASKELL (**13**) showed that the equilibrium ratio of similarly charged inorganic and organic phosphate ions is constant over the entire pH range of enzyme activity.

The phenomena of essential sulfhydryl groups in enzymes and of the inhibition of enzymes by sulfhydryl reagents have been much investigated and the subject has been discussed in reviews by OLCOTT and FRAENKEL-CONRAT (**20**), by SINGER (**28**), by MASSART (**17**) and more recently in a comprehensive review by BARRON (**2**). Much of the pioneer work in this field was carried out by RAPKINE (**23, 24**), who used chiefly the reactions between sulfhydryl groups and the alkylating agents, iodoacetate and iodoacetamide, for the detection and study of essential sulfhydryl groups in enzymes. It has since been found that these reagents do not react with all sulfhydryl groups, and their specificity has been questioned (**2**). A number of other reagents are now preferred for the study of sulfhydryl groups in enzymes. They include oxidizing agents (such as iodine, oxidized glutathione, iodosobenzoate, and porphyrindin), and mercaptide forming agents (such as heavy metals, *p*-chloromercuribenzoate, and organic arsenicals). These two groups of reagents are of particular interest because their reactions with sulfhydryl groups are reversible. Thus enzymes inactivated by these reagents may frequently be reactivated by reducing agents or thiols. The reactivations help to confirm the mode of action of the inhibitors.

An apparently selective inhibition of amylase in the presence of phosphorylase by iodine was observed by HANES (**11**) in aqueous extracts of peas. NAKAMURA and MARUO have (**19**) described a similar technique for detecting phosphorylase in the presence of amylases. They used mercury compounds as selective inhibitors of the amylases in enzyme preparations

from several plants. Both the iodine-potassium iodide reagent and the mercury compounds are known to react with sulfhydryl groups. Thus the qualitative observations suggest that the activity of the amylase systems is dependent on the integrity of sulfhydryl groups within the enzyme molecules, while that of the phosphorylases is independent of sulfhydryl groups. The observations would be explained also, if the phosphorylases contain sulfhydryl groups which, though essential for enzyme activity, are less reactive chemically than those of the amylases. The possibility of obtaining complete inhibition of the locust bark amylase without inhibiting the phosphorylase was explored. Iodine, iodoacetate, *p*-chloromercuribenzoate, phenylmercuric chloride, and phenylmercuric nitrate have been used as sulfhydryl reagents. Cysteine has been used in efforts to reverse the enzyme inhibition produced by these reagents. The results of the experiments with sulfhydryl reagents on the amylase and phosphorylase systems of the living bark of the black locust tree are reported here as a part of the characterization of these enzyme systems.

Materials and methods

TISSUE EXTRACTS.—The dead outer bark of black locust tree logs was removed and the inner, living bark was ground off with a motor-driven wire brush wheel. Samples of the freshly shredded tissue were soaked in three to four volumes of cold distilled water (5° C) for 2–3 hours with periodic trituration and the tissue was squeezed to remove as much as possible of the water extract. The residual tissue was washed with two volumes of cold distilled water, the washings combined with the original extract. The extract was filtered to remove tissue debris.

Amylase activity in the extract was demonstrated qualitatively by incubating aliquots with starch solution and periodically testing a few drops of the reaction mixture with iodine-potassium iodide reagent for evidence of starch degradation. The tests indicated that a highly active amylase system occurs in this tissue.

Qualitative tests for phosphorylase activity were made by incubating aliquots of the tissue extracts with glucose-1-phosphate and a trace of starch primer. At intervals of approximately five minutes a few drops of the test solution were treated with iodine-potassium iodide reagent for evidence of starch synthesis. The amylase activity of the crude tissue extracts was sufficiently great to destroy the starch primer and any synthetic starch and the tests remained negative. Following the method of HANES (**11**), however, it was found that low concentrations of iodine-potassium iodide reagent added to the enzyme-substrate mixture inactivated the amylase system to a marked degree, presumably by reaction with sulfhydryl groups, while leaving strong phosphorylase activity. In this manner it was possible to obtain intense blue color tests, similar to those given by the linear component of starch, for the synthetic polysaccharide with the iodine-potassium iodide reagent.

AMYLASE PREPARATION.—The proteins from the tissue extract were fractionally precipitated by addition of cold saturated neutral $(NH_4)_2SO_4$. The precipitates were taken up in McIlvaine citrate-phosphate buffer, pH 6.6, and dialyzed at 5° C during three days against water followed by three changes of the buffer, pH 6.6, diluted 1 to 10. The fraction precipitated between 36 and 44% saturation with $(NH_4)_2SO_4$ showed the greatest amylase activity and was used for the amylase experiments.

Estimates of amylase activity were obtained by measuring the increase in reducing sugars after aliquots of the enzyme preparation were incubated with a standard solution of starch. The reducing power was measured by oxidation with alkaline ferricyanide and titration of the resulting ferrocyanide with 0.01 *N* ceric sulfate according to the method of HASSID (**14**). Enzyme action in the sample aliquots was stopped by transferring them to test tubes containing the alkaline ferricyanide reagent. Enzyme activity is expressed as the volume of 0.01 *N* ceric sulfate equivalent to the reducing sugars produced per mg. of nitrogen in the enzyme preparation during the time the enzyme was allowed to act.

PHOSPHORYLASE PREPARATION.—Since the polysaccharide synthesizing action of phosphorylase is inhibited by inorganic phosphate, enzyme preparations containing phosphate buffer were not used for the phosphorylase experiments. The total proteins were precipitated from the tissue extract by addition of 600 gm. solid $(NH_4)_2SO_4$ per liter of extract, separated by filtration, and taken up in 0.05 M citrate buffer, pH 6.7. The solution was dialyzed for 40 hours against cold, flowing distilled water and for an additional 45 hours against two changes of the citrate buffer. Sediment was removed by centrifugation and the supernatant was used for the phosphorylase experiments. This preparation contained amylase as well as phosphorylase but, where starch primer was added and the initial rates of inorganic phosphate formation from the glucose-1-phosphate substrate were determined, the amylolytic action was considered non-determinant in the studies of phosphorylase activity.

Phosphorylase activity was usually estimated by measuring the rate at which the release of inorganic phosphate from glucose-1-phosphate was catalyzed by the enzyme preparations. Unless 0.1% or more of soluble starch was added to the enzyme-substrate mixtures the reaction was characterized by an induction period. Because the reaction approaches a state of equilibrium as inorganic phosphate accumulates, the initial rate of increase in concentration of inorganic phosphate was generally used as the measure of phosphorylase activity. In one experiment reported here phosphorylase activity was demonstrated by measuring the disappearance of inorganic phosphate and the production of glucose-1-phosphate when an aliquot of the enzyme was incubated with starch and phosphate buffer.

ANALYSIS FOR PHOSPHORUS COMPOUNDS.—Inorganic phosphate and glucose-1-phosphate were estimated by a modification of the method of LOWRY and LOPEZ (**16**). An aliquot of 1–3 ml. from the enzyme-substrate mixture

was mixed with three volumes of 8 to 10% (w/v) trichloracetic acid in a small centrifuge tube. The solution was adjusted to pH 4.0 with 1 *N* sodium acetate, the amount of sodium acetate required having been determined by titrating a separate sample to a pale green color with brom cresol green. These neutralized samples were heated in a boiling water bath for one minute, cooled, and centrifuged. Aliquots of supernatant were transferred to 25-ml. volumetric flasks and to each flask 5 ml. of 0.1 M acetate buffer, pH 4.0, 2.5 ml. of 1% ascorbic acid in acetate buffer, 2.0 ml. of 2.5% ammonium molybdate, and distilled water to dilute to 25 ml. were added. The color was allowed to develop for 5–15 minutes and the optical density was measured at 670 mμ in a Coleman Model 11 photoelectric spectrophotometer. Blank samples were obtained by processing aliquots of the enzyme-substrate mixture immediately after the glucose-1-phosphate was mixed with the enzyme. The inorganic phosphate equivalent to the observed optical density was estimated by reference to a standard curve.

When estimating the concentration of inorganic phosphate plus glucose-1-phosphate (7-minute acid hydrolyzable phosphate) the trichloracetic acid was used to hydrolyze the phosphate ester. The acid-treated aliquots were placed in a boiling water bath for 7 minutes. The hydrolyzed samples were then cooled, neutralized to pH 4.0 with 1 *N* sodium acetate and diluted to a definite volume (*e.g.*, 25 ml.) in volumetric flasks. These diluted samples were then centrifuged and aliquots of the supernatant were analyzed for inorganic phosphate according to the method previously described.

Experimental results

Effects of temperature, pH, and other environmental conditions upon the activities of locust bark amylase and phosphorylase

Optimal temperature for amylase activity.—Figure 1 shows the effect of temperature on the locust tree amylase. Incubation times of 27 minutes (curve A) and 64 minutes (curve B) were employed. During the shorter incubation period maximal hydrolysis occurred at or above 55° C. With the longer incubation period there was definite evidence of enzyme inactivation at 55° C, with maximal hydrolysis occurring at 50° C.

In figure 2 the data of the 27-minute incubation period experiment are examined graphically for compliance with the Arrhenius equation which may be written,

$$\log k = C - \frac{A}{2.3\,RT}$$

where k represents the rate of starch hydrolysis at absolute temperature, T, C is a constant, and A is the activation energy. A plot of log k against the reciprocal of T should give a straight line with slope $\frac{-A}{2.3\,R}$ and thence the value of A may be estimated from the observed value of the slope. The

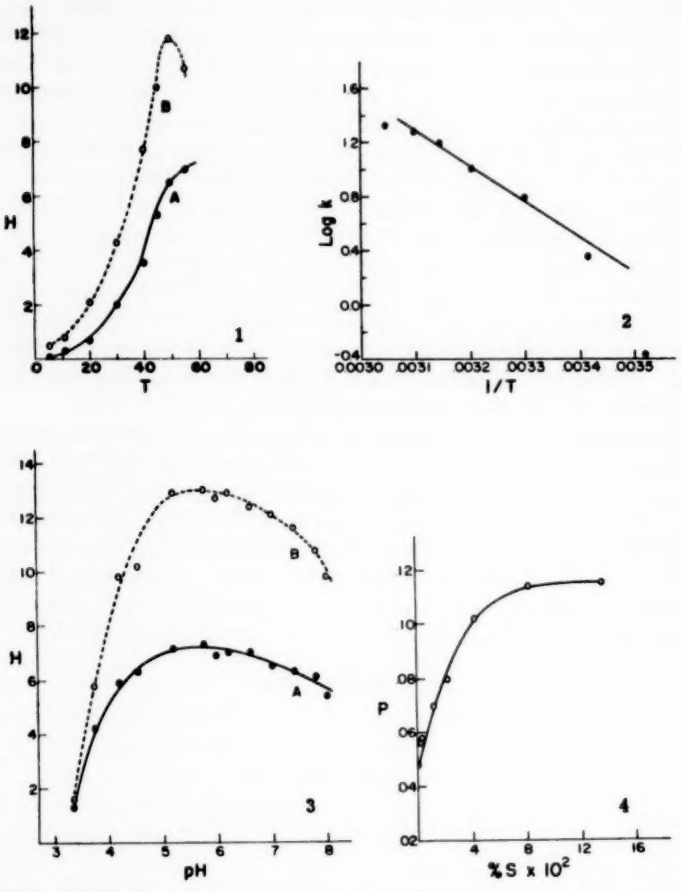

FIG. 1. Effects of temperature, T (°C), on hydrolysis, H, of starch by locust tree amylase, at pH 6.6. H is expressed as ml. of 0.01 *N* ceric sulfate equivalent to increase in reducing sugars per mg. of nitrogen in the aliquot of enzyme preparation employed. Curves A and B refer to reaction times of 27 and 64 minutes, respectively. Each sample contained 1 ml. of enzyme preparation, 5.0 ml. of 1% soluble starch and 3 ml. of McIlvaine citrate-phosphate buffer, pH 6.6.

FIG. 2. Graphic estimation of the activation energy of the locust tree amylase hydrolysis of starch from the slope of the plot of the logarithms of reaction rates, K (glucose equivalent formed in identical enzyme substrate aliquots after 27 minutes incubation), versus the reciprocal of the temperature, T (in degrees absolute).

FIG. 3. Effects of pH on the hydrolysis, H (units same as in fig. 1), of starch by locust tree amylase. Temperature of incubation was 39° C. Curves A and B refer to reaction times, 30 and 60 minutes, respectively. Each sample contained 1 ml. of enzyme preparation, 3 ml. of McIlvaine citrate-phosphate buffer, and 5 ml. of 4% soluble starch.

FIG. 4. Effects of soluble starch (primer) concentrations, % S, on the amount of inorganic phosphorus, P, released from glucose-1-phosphate during 30 minutes incubation

experimental data appear to comply with the equation at intermediate temperatures (30–50° C). The activation energy, A, calculated from the line drawn through four points, is 12,000 calories per mole.

Optimal pH for amylase activity.—The manner in which the activity of the amylase at 39° C and incubation times of 30–60 minutes was affected by pH, is shown in figure 3. There was low enzyme activity and probable inactivation of the enzyme at pH 3.4. There is a wide plateau of maximal activity in the region between pH 5.3 and 6.0 which suggests the probability of the presence of two or more amylases having different pH optima.

Effect of starch concentration on phosphorylase activity.—In the absence of sufficient starch to fully "prime" the reaction the rate at which release of inorganic phosphate from glucose-1-phosphate was catalyzed by the locust tree phosphorylase was characterized by an induction period. Amylolytic splitting of the suboptimal amount of polysaccharide with a consequent increase in the number of terminal non-reducing units may account for an autocatalytic effect which followed. The induction period was eliminated by adding sufficient soluble starch. In figure 4 the effect of the concentration of the starch primer on the amount of inorganic phosphate released during 30 minutes at 30° C is shown. A maximum of activity was approached when the concentration of starch in the sample mixtures exceeded 0.1%.

Effect of temperature on phosphorylase activity.—In figure 5 the effects of temperatures, ranging from 5–45° C, on the amounts of inorganic phosphate liberated from glucose-1-phosphate during 45 minutes by locust tree phosphorylase are compared. While in this experiment the activity was greatest at the highest temperature employed, the curve appeared to be approaching a maximum in this temperature region. In a subsequent experiment a maximal activity was obtained at 50° C while at 55° C the enzyme was rapidly inactivated.

Equilibrium of the phosphorylase reaction.—The reversibility of the reaction catalyzed by the locust tree phosphorylase is demonstrated in figure 6. Experiments were made at 30° C and pH 6.7, and equilibrium was approached from either direction. Although there was no evidence of interference by a phosphatase sensitive to fluoride, NaF was added as a precautionary measure in these experiments. When glucose-1-phosphate was the substrate (fig. 6 A) the observed final ratio of inorganic phosphate to glucose-1-phosphate was 4.1. For the experiment with starch and inorganic phosphate as initial substrate (fig. 6 B) the final ratio of inorganic phosphate to glucose-1-phosphate was 5.9. The first value, 4.1, agrees more closely with the equilibrium ratio at this pH reported for other phosphorylase systems (**9, 12**). It is possible that in the latter case amylolytic de-

time at pH 6.6 and 30° C by locust tree phosphorylase. P is expressed as mg. phosphorus released per mg. of nitrogen in the aliquot of enzyme preparation employed. Each sample contained 0.5 ml. of phosphorylase preparation, 0.3 ml. of 1.5% dipotassium glucose-1-phosphate in 0.05 M citrate buffer, pH 6.6, and an additional 0.3 ml. of the citrate buffer solution containing from 0.0 to 0.5% soluble starch.

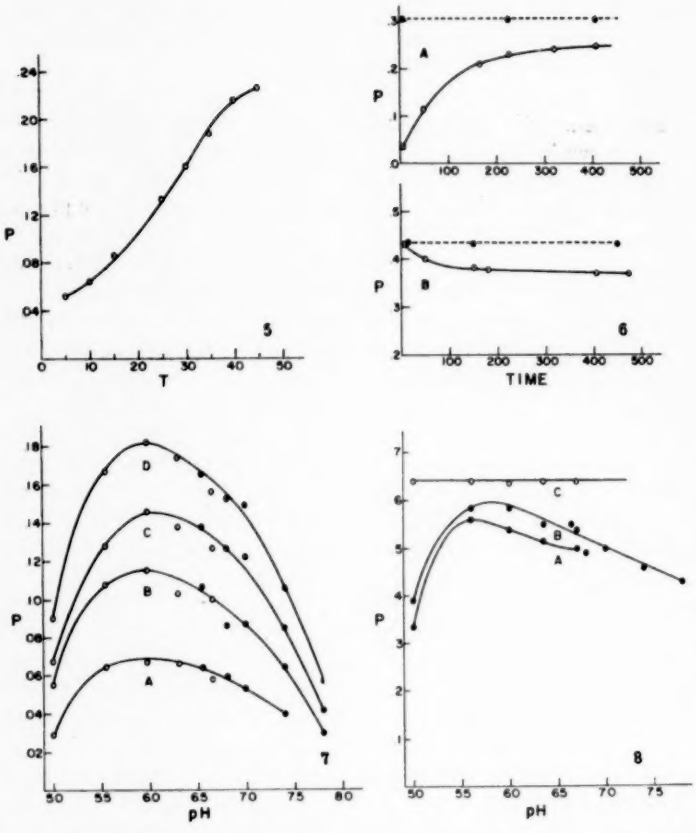

FIG. 5. Effect of temperature on locust tree phosphorylase activity at pH 6.6. Amounts of inorganic phosphate, P (units same as in fig. 4), released from glucose-1-phosphate during a 45-minute incubation time at various temperatures. Each sample contained 0.5 ml. of the enzyme preparation, 0.3 ml. of 1.5% dipotassium glucose-1-phosphate in 0.05 M citrate buffer, pH 6.6, and 0.3 ml. of 0.5% soluble starch in citrate buffer. The whole samples were used for the analyses.

FIG. 6. Approach to equilibrium from either direction of the reaction catalyzed by locust tree phosphorylase at 30° C. Dashed lines represent concentration of inorganic plus 7-minute hydrolyzed phosphate. Full line represents concentration of inorganic phosphate. P is in same units as in figure 4. In figure 6 A the samples contained 8 ml. of enzyme preparation, 3 ml. of 1.4% dipotassium glucose-1-phosphate in 0.5 M citrate buffer, pH 6.7, 3.0 ml. of 1% soluble starch in citrate buffer and 1.0 ml. of 1.0 M sodium fluoride. In figure 6 B the samples contained 10 ml. of enzyme preparation, 3.0 ml. of 1.0 M sodium fluoride in 0.05 M citrate buffer, pH 6.7, 2.0 ml. of 0.1 M phosphate buffer, pH 6.6, and 10.0 ml. of 4% soluble starch.

FIG. 7. Effects of pH on net rate at which the release of inorganic phosphate, P (units same as in fig. 4), from glucose-1-phosphate, is catalyzed by locust tree phosphorylase. Temperate was 30° C. Curves A, B, C, and D refer to 15, 30, 45, and 60

struction of the starch substrate stopped further phosphorolysis, short of equilibrium.

EFFECT OF pH ON PHOSPHORYLASE ACTIVITY.—In figure 7 the effect of pH on the early progress of the reaction catalyzed by the locust tree phosphorylase at 30° C is shown. The substrate for each sample was prepared by adjusting 1.5 ml. of an approximately 1.5% solution of dipotassium glucose-1-phosphate to the required pH with hydrochloric acid and diluting to 4 ml. with distilled water. Each substrate sample was mixed with 5.0 ml. of 0.5 M citrate buffer or veronal buffer (**18**) of the required pH, 1.0 ml. of 1% soluble starch and finally 2.0 ml. of enzyme preparation. When incubated for periods of 30, 45, or 60 minutes (curves B, C, and D, respectively) the greatest net liberation of inorganic phosphate occurred at pH 6.0. For a 15-minute incubation (curve A) the accumulation of inorganic phosphate was as great at pH 6.3 as at pH 6.0. Equal concentrations of inorganic phosphate at pH 6.0 and 6.3 represent a closer approach to equilibrium at pH 6.3 (**12**). Therefore, the apparent optimum at pH 6.0 may not indicate a maximum of enzyme activity. It appears that this phosphorylase is most active at some pH between 6.0 and 6.5.

Analyses of aliquots from the previously described samples after prolonged incubations (10–21 hours) showed that pH dependent equilibrium ratios were approached. From figure 8 it is seen that between pH 5.6 and 7.8 the final concentration of inorganic phosphate decreased with rising pH. This is consistent with the expected higher equilibrium ratios at lower pH levels (**9, 12**). The low level of inorganic phosphate released at pH 5.0 may have been due to a combination of low enzyme activity and enzyme inactivation. It was not possible to estimate the actual equilibrium ratios from these results because the sum of inorganic plus 7-minute hydrolyzable phosphate was determined only during the early stages of the reaction and prior to possible volume alteration due to evaporation of water during the prolonged incubations.

IDENTIFICATION OF PRODUCTS OF ENZYME REACTIONS BY PAPER CHROMATOGRAPHY

The products of the reactions catalyzed by the locust tree amylase and phosphorylase systems were separated and identified by descending paper chromatography (**21**). The solvent combinations used were a 40% (by volume) *n*-butanol, 10% ethanol and 50% water mixture or a 40% *n*-butanol, 10% propionic acid and 50% water mixture. The products were detected

minutes incubation times respectively. Open circles refer to experiments conducted in citrate buffers, filled circles to those in veronal buffers.

FIG. 8. Effects of pH on final concentrations of inorganic phosphate and glucose-1-phosphate in presence of locust tree phosphorylase at 30° C. Curves A and B refer to final concentration of inorganic phosphate, P (units same as in fig. 4), after 600 and 1300 minutes incubation time respectively. Curve C refers to sum of inorganic and 7-minute hydrolyzable phosphorus present.

on the paper chromatograms by spraying with either a 1:1 mixture of freshly prepared 2 *N* silver nitrate and concentrated NH_4OH or a mixture containing 2 gm. of trichloracetic acid, 0.2 gm. of *p*-amino dimethyl aniline monohydrochloride and 50 ml. of water. The products were identified by comparison with simultaneously developed chromatograms of mixtures of known sugars. Unhydrolyzed starch could be identified at the starting point on all chromatograms of the enzymatic digests.

With prolonged incubations (*e.g.*, 19 hours) the chief products of the action of the amylase on starch were found to be maltose and glucose, with maltose predominating. There were also diffuse spots of low R_F values indicating dextrins. With shorter incubation periods (*e.g.*, 6 hours) the only products detected were maltose and dextrins. The results were identical whether citrate or phosphate buffers were employed in the reaction mixtures.

The products of the action of crude phosphorylase preparations on glucose-1-phosphate were starch, dextrins, and maltose. The maltose and dextrins probably resulted from the action of the amylase, which is always present, on the synthetic starch.

The action of sulfhydryl reagents upon the amylase and phosphorylase enzymes

The inhibition of amylase.—Attempts to demonstrate inhibition of the locust tree amylase by iodoacetate or ferricyanide were unsuccessful. However, the locust tree amylase was inhibited by iodine-potassium iodide

TABLE I

Inhibition of amylase.

Inhibitor	Ml. of .01 *N* $Ce(SO_4)_2$ per mg. N_2		
	30 min.	60 min.	90 min.
Control (no inhibitor added)	4.55	9.45	14.1
2.5×10^{-5} M I_2–7.5×10^{-5} M KI	.88	1.55	2.3
0.5×10^{-4} M I_2–1.5×10^{-4} M KI	....	.25	.25
0.75×10^{-4} M I_2–2.3×10^{-4} M KI	.20	.15	.15
1.0×10^{-4} M I_2–3.0×10^{-4} M KI	....	....	....
2.5×10^{-4} M C_6H_5-Hg-Cl	.35	.6	.9
2.0×10^{-4} M C_6H_5-Hg-NO_3	1.05	1.70	2.55
2.5×10^{-4} M p-Cl-Hg-C_6H_4-COOH	.35	.3	.4

Inhibition of locust tree bark amylase at 35°C and pH 6.3 by iodine-potassium iodide reagent, phenylmercuric chloride (C_6H_5-Hg-Cl), phenylmercuric nitrate (C_6H_5-Hg-NO_3) and *p*-chloromercuribenzoate (*p*-Cl-Hg-C_6H_4-COOH). In each experiment the sample contained amylase preparation equivalent to 10 mg. protein nitrogen, 5 ml. of McIlvaine citrate phosphate buffer, inhibitor in amount to yield the final concentration shown and distilled water to give a total volume of 20 ml.

reagent, phenylmercuric chloride, phenylmercuric nitrate, or *p*-chloromercuribenzoate. Some representative data are shown in table I. In all experiments the buffer, substrate, inhibitors, and distilled water were mixed before the enzyme was added. The results indicate that 0.5×10^{-4} M iodine in 1.5×10^{-4} M potassium iodide or approximately 2.5×10^{-4} M concentrations

of the mercury compounds produced almost complete inhibition of the enzyme. The similarity in the effective equivalent concentrations suggests that the different inhibitors react with the same groups in the enzyme molecules.

Reversal of amylase inhibition.—In figure 9 are shown the results of an experiment in which inhibition of the locust tree amylase by phenylmercuric nitrate was reversed with cysteine. Analyses of aliquots from sample C subsequent to the cysteine addition were corrected for the reducing action of the cysteine. Inhibition of the amylase by phenylmercuric nitrate was completely reversed by the cysteine. Similar reactivations by cysteine were observed when *p*-chloromercuribenzoate and phenylmercuric chloride were the inhibitors used.

The inhibition of phosphorylase.—An attempt to demonstrate inhibition of the black locust tree phosphorylase by iodoacetate was, as in the case of the amylase, unsuccessful. However, as is shown in figure 10, the phosphorylase was inhibited by iodine-potassium iodide reagent. The excess starch added in these experiments prevented the amylase present from influencing the phosphorylase action.

In qualitative experiments with crude enzyme preparations, sulfhydryl reagents had appeared to give selective inhibition of the amylase. Therefore, to determine whether complete inhibition of the amylase could be achieved without any inhibition of the phosphorylase, the effects of different concentrations of the iodine-potassium iodide reagent on the amylase and phosphorylase activities of a single enzyme preparation were compared. The results, expressed as per cent. inhibition of enzyme activity, are shown in figure 11. The percentage inhibition of the amylase activity was estimated from the increase in reducing carbohydrate in the different samples after incubating for 40 minutes at 35° C. The percentage inhibition of the phosphorylase was estimated by comparing the quantities of inorganic phosphate liberated from glucose-1-phosphate during the 32-minute period between the first and second samplings in the preceding experiment (fig. 10). From figure 11 it is evident that it would be possible to obtain complete inhibition of the amylase with iodine-potassium iodide reagent while considerable phosphorylase activity remained. However, it appears to be impossible to obtain complete inhibition of one enzyme without partial inhibition of the other.

Reversal of phosphorylase inhibition.—Figure 12 shows the results of an experiment in which the phosphorylase was inhibited with phenylmercuric nitrate and reactivated with cysteine. In similar experiments it was found that the phosphorylase could be inhibited with phenylmercuric chloride and reactivated with cysteine. It was also shown that the enzyme was inhibited by *p*-chloromercuribenzoate, but no attempt was made to demonstrate reversal of this inhibition.

Discussion

The observed variations in activity of an enzyme under different conditions of temperature and pH can represent the combined effects of inacti-

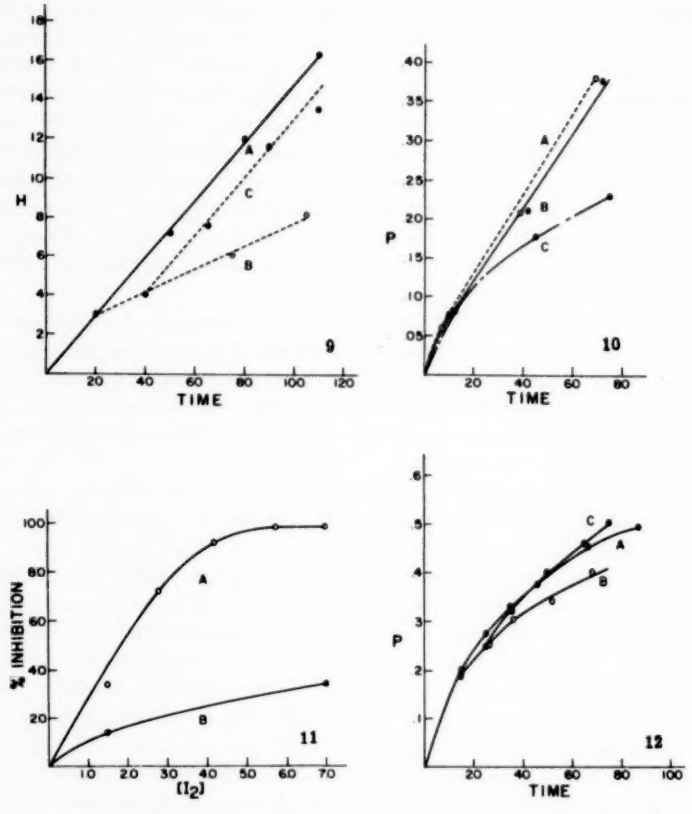

FIG. 9. Reversible inhibition of locust tree amylase with phenylmercuric nitrate and cysteine at 35° C and pH 6.3. Increase in hydrolysis, H (units same as in fig. 1), versus time (minutes) of incubation. Curve A—no inhibitor added. Curve B—inhibitor added at final concentration of 2×10^{-4} M after 20 minutes. Curve C—inhibitor added as in curve B followed 20 minutes later by addition of cysteine at final concentration of 1.65×10^{-2} M. Each sample contained amylase preparation equivalent to 10 mg. of protein nitrogen and 0.15 gm. wheat starch. Citrate buffer concentration in the final solutions (25 ml.) was 0.02 molar.

FIG. 10. Inhibition of locust tree phosphorylase, at 30° C and pH 6.7, with iodine-potassium iodide reagent. P is expressed in same units as in figure 4. Time is in minutes. Curve A is a control with starch added at 6 minutes after zero time. With curve B, starch was added at 8 minutes and inhibitor at a final concentration of 1.9×10^{-5} M $I_2 + 5.4 \times 10^{-5}$ M KI was added at 9 minutes after zero time. For curve C, starch was added at 11 minutes and inhibitor at final concentration 0.9×10^{-4} M I_2 $+ 2.7 \times 10^{-4}$ M KI was added at 12 minutes after zero time. Each sample contained enzyme preparation equivalent to 1.45 mg. of protein nitrogen, 1.0 mg. of soluble starch, and 7.5 mg. of glucose-1-phosphate (neutralized) in 5.5 ml. of 0.01 M citrate buffer. Inorganic phosphorus is corrected for reagents blank at zero time. Zero time was taken from the moment that the enzyme and glucose-1-phosphate were mixed.

vation and actual changes in activity. In the experiments reported here no attempt was made to differentiate between these two phenomena.

Measurements of the activity of amylases based on the increase in reducing sugars do not, of course, identify or classify the mode of action of the enzyme or enzymes (*e.g.*, α or β). The appearance of high concentrations of maltose while considerable high molecular weight material remained in the enzyme substrate mixtures suggests that an enzyme of the β-amylase type was present. Since the formation of glucose from starch is believed to be characteristic of α-amylase (**22**), the chromatographic detection of glucose after long incubation suggests that an α-amylase was also present. Thus, it is probable that amylases of both the α and β types occur in the locust tree tissue.

The action of the locust tree phosphorylase appears to be similar to that of other phosphorylases from plant and animal sources (**8, 9, 11, 12**). The synthesis of polysaccharide is dependent upon the presence of a small amount of polysaccharide to act as a primer. The reaction is reversible and approaches an equilibrium which is independent of the concentration of polysaccharide but varies with pH.

The inhibitions of these enzymes by sulfhydryl reagents indicate that both the amylase and the phosphorylase systems of the black locust tree are dependent on the integrity of sulfhydryl groups. Failure of efforts to inhibit the enzymes with iodoacetate or ferricyanide do not discredit this conclusion because some protein sulfhydryl groups do not react readily with these reagents (**2**). Since concentrations of the iodine-potassium iodide reagent which gave complete inhibition of the amylase gave only partial inhibition of the phosphorylase, it appears that the sulfhydryl groups of the phosphorylase are not as reactive as those of the amylase. However, there is not sufficient difference in reactivity to permit quantitative inhibition of the amylase without some concomitant inhibition of the phosphorylase. There is a possibility that the difference in reactivity with sulfhydryl reagents may

FIG. 11. Comparison of inhibition of locust tree amylase and phosphorylase in a single crude enzyme preparation with varying concentrations of iodine–potassium iodide reagent, concentration of I_2 in the incubating solutions is given as milliequivalents $\times 10^{-4}$ of iodine added per milligram of nitrogen in the aliquot of enzyme preparation used. Curves A and B refer to amylase and phosphorylase activities, respectively. In the amylase inhibition experiments each sample contained tissue extract equivalent to 7.2 mg. protein nitrogen, 150 mg. wheat starch in total volume of 25 ml. of 0.01 citrate buffer, pH 6.7. Phosphorylase inhibition results are taken from data of figure 10.

FIG. 12. Reversible inhibition of locust tree phosphorylase, at 30° C and pH 6.7, with phenyl mercuric nitrate and cysteine. Units are same as for figure 10. Curve A is a control. In curves B and C phenyl mercuric nitrate (final concentration $= 1 \times 10^{-4}$ M) was added at 15 minutes after zero time. In curve C cysteine (final concentration $= 1.1 \times 10^{-2}$ M) was added at 27 minutes after zero time. Each sample contained phosphorylase preparation equivalent to 4.3 mg. protein nitrogen, 20 mg. of soluble starch, and 30 mg. of glucose-1-phosphate in 12 ml. final volume of 0.01 citrate buffer. Zero time is taken as moment at which enzyme-starch mixture is added to glucose-1-phosphate solution.

play a role in the regulation of metabolic processes. Natural sulfhydryl reagents acting as inhibitors of specific enzymes may be involved, for example, in the seasonal changes in the chemical constitution of the protoplasm (**27**). The phosphorylase inhibitor reported by ARREGUIN-LOZANO and BONNER (**1**) may be such a sulfhydryl reagent, being elaborated in potatoes during storage at warm temperatures and absent when the potatoes are stored at cold temperatures.

These characterization studies on the amylase and phosphorylase systems of the living bark of the black locust tree have yielded no definite clues as to whether or how these enzymes may be involved in the temperature sensitive reversible deposition (or mobilization) of starch which occurs in these tissues and which apparently play a minor role in the abilities of the locust bark to withstand injury by freezing.

Summary

Amylase and phosphorylase enzyme systems have been detected in the living bark tissue of the black locust tree.

The optimal temperature for the amylase system during a short incubation period (27 minutes), at pH 6.6, was greater than 55° C. With a longer incubation time (64 minutes) there was pronounced inactivation of the enzyme at 55° C and maximum activity was observed at 50° C. The activation energy for the amylase catalyzed reaction was approximately 12,000 calories per mole. The optimal pH range in citrate-phosphate buffer for the amylase system at 39° C was 5.3–6.0. The products of hydrolysis of starch by the locust tree amylase were maltose, glucose, and dextrins.

Synthesis of polysaccharide from glucose-1-phosphate by the locust tree phosphorylase was dependent on the presence of a small amount of polysaccharide primer. The reaction catalyzed by the locust tree phosphorylase was reversible and approached an equilibrium ratio of inorganic phosphate to glucose-1-phosphate which was independent of the concentration of starch. This ratio was lowest at the highest pH values. The optimal temperature for action of the locust tree phosphorylase during 45 minutes, at pH 6.6, was approximately 50° C. The optimal pH in citrate buffer, as revealed by the initial rate of release of inorganic phosphate from glucose-1-phosphate at 30° C, was approximately 6.3.

The amylase system of the living bark of the black locust tree was inhibited by iodine-potassium iodide reagent, *p*-chloromercuribenzoate, phenylmercuric chloride, and phenylmercuric nitrate, but was not inhibited by iodoacetate or ferricyanide. The inhibitions produced by *p*-chloromercuribenzoate, phenylmercuric chloride, and phenylmercuric nitrate were readily reversed by an excess of cysteine.

The phosphorylase of the living bark of the black locust tree was inhibited by iodine-potassium iodide reagent, *p*-chloromercuribenzoate, phenylmercuric chloride, and phenylmercuric nitrate, but it was not inhibited by iodoacetate. The inhibitions produced by phenylmercuric chloride and phenylmercuric nitrate were readily reversed by cysteine.

In experiments with crude enzyme preparations containing both amylase and phosphorylase it was shown that concentrations of the iodine-potassium iodide reagent which gave complete inhibition of the amylase gave only partial inhibition of the phosphorylase.

These studies indicate that both the amylase and the phosphorylase systems of the black locust tree are dependent on the integrity of sulfhydryl groups. However, the essential sulfhydryl groups of the phosphorylase are not as chemically reactive as those of the amylase. It is suggested that differential sensitivity of enzymes to natural sulfhydryl reagents may be involved in the regulation of metabolic processes of plants and particularly in controlling the seasonal changes in composition of the protoplasm of the black locust tree living bark.

The authors are indebted to the Herman Frasch Foundation for Chemical Research for a grant of funds in support of research on the problem of winter hardiness in plants. The present paper constitutes a report on a part of this research.

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THE INFLUENCE OF SOIL PHYSICAL CONDITION DURING THE FLOWERING PERIOD ON NECTAR PRODUCTION IN SNAPDRAGON

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Received August 29, 1952

During the past few years the problem of nectar secretion has acquired increased significance because of its bearing on pollination and seed and fruit set. Although optimal conditions for nectar yield have not been clearly defined, there is evidence that an abundant supply of carbohydrate is important. A correlation between nectar yield and quantity of solar radiation has been found for red clover (**7**), and in horse chestnut yield has been shown to be related to the magnitude of the carbohydrate supply to the flower (**9**). Little is known about the mechanism. Secretion of nectar involves a transfer of sugars against a concentration gradient, and appears to be dependent on metabolic activity in the nectary tissues (**6**). Nectar concentration ranges characteristic of species have been shown by AGTHE (**1**) to be related to the proportions of xylem and phloem in the vascular supply to the nectaries. The manner in which volume and concentration are controlled within the individual plant, however, and the mechanism by which the necessary energy is supplied, are still obscure.

JOHNSON (**4**) has noted that in New Zealand white clover consistently produces more nectar on sandy soils than on clay soils, except in drought years. This effect may be attributable in part to the action of such soil physical factors as moisture level, temperature, and aeration during the flowering period. The influence of these factors has been investigated in the present study under controlled conditions. Snapdragon (*Antirrhinum majus* L.) has been used as a test plant because of its high nectar production and suitability for winter greenhouse culture.

Materials and methods

Although it was appreciated that the soil environment during early plant growth may greatly influence nectar secretion by conditioning plant growth and composition, it was desired in this instance to limit the study to the relatively short-term effects during the flowering period. All plants, therefore, were given similar treatment prior to flowering. Hybrid F_1 plants were used to avoid the masking of treatment effects by genetic variation. Flowers used for nectar assay were collected as soon as possible after reaching full size. The centrifuge method (**8**) was used for nectar measurement. Flowers were inverted in calibrated glass capillary tubes after clipping off the upper part of the corolla and centrifuged for six minutes at several hundred $\times$ g. The sugar concentration of the centrifugate was read

with an Abbe refractometer, and sugar weight yields were derived from volume and concentration values. Weight of total nectar sugar was found to be less variable than nectar volume. For precise concentration comparisons, refractometer readings were taken on samples drawn from flowers with glass capillaries, thus avoiding possible errors due to the dilution of the nectar during centrifugation with extraneous water from floral tissues. All yield data were treated statistically.

The effect of soil moisture level on nectar yield

The soil water study was designed to measure the effect of soil water level over a wide range on both quantity and sugar concentration of nectar. Plants were grown in pots containing Burford loam, chosen because it dries relatively slowly under winter greenhouse conditions. The approximate textural composition of this soil is: sand, 32%; silt, 49%; and clay < 0.002 mm., 19%. A plaster-of-Paris Bouyoucos block was placed on edge in each pot at the 3-inch level. Moisture readings were taken on the blocks with the Bouyoucos resistance bridge and available moisture meter once or twice daily as flowers were collected for nectar assay.

When flower buds were well developed, plants were divided into three groups. The first group was kept at saturation (53.5%) and the second at moisture equivalent (19.6% and approximating closely to the field capacity), with daily additions of water. In the third group, the soil was allowed to dry out to the permanent wilting percentage (9.1%), then brought back to the moisture equivalent with a weighed increment of water. This hydraulic cycle was repeated throughout the flowering period.

The first samples for nectar assay were collected on the fifth day after the initiation of treatments. By the seventh day all plants in saturated soil were severely wilted, and by the fourteenth day their root systems had rotted. Temporary wilting on bright days, with recovery of turgor at night, was common in the drying soil treatment. Flower size was greatly reduced in the saturation treatment, though flower production continued as long as in the other treatments. There was no appreciable difference in flower size between moisture equivalent and drying cycle treatments.

An examination of the curve relating available moisture to total soil moisture revealed two inflection points, suggestive of a slight variation in water availability over the available moisture range, at the 75% and 25% available water levels. Nectar yield data from the drying cycle treatment were therefore divided into three groups: 75 to 100%, 25 to 75%, and less than 25% available water. Table I contains data on sugar weight yields for the different treatment groups, expressed as a percentage of the yield for the control group (moisture equivalent) for the same period, and nectar concentrations. Differences in values for the control, or moisture equivalent group, in the various comparisons are due to the fact that the comparisons encompassed slightly different periods. Each comparison involved a sampling period of several days, and, in all, some 400 flowers were as-

TABLE I

MEAN SUGAR YIELDS AND CONCENTRATIONS OF SNAPDRAGON NECTAR AT VARIOUS LEVELS OF SOIL MOISTURE.

Treatment	Mean nectar sugar yield per flower expressed as % of control (moisture equivalent)	Mean concentration (%)	
		Treated	Control (ME)
Saturation	50.7**	64.9	57.1*
75–100% available water	85.7*	47.7	49.8
25–75% available water	75.4**	40.4	39.9
< 25% available water	82.3**	54.8	46.4*

*Mean difference significant at 5% level.
**Mean difference significant at 1% level.

sayed. In order to avoid the effect on concentration readings of differential dilution with varying turgor in the floral tissues, concentration comparisons were based on samples pipetted directly from the flowers rather than on centrifuged samples. Nectar volume yields, which are not reported here, closely paralleled sugar weight yields.

Deviations in either direction from the optimal soil moisture level—the moisture equivalent—led to a reduction in nectar yield. The greatest reduction occurred at the extremes of the moisture range, but even severely wilted plants invariably secreted some nectar. Nectar concentration values varied very little in the intermediate section of the moisture range, but at the extremes the nectar was relatively concentrated.

In view of the fact that snapdragon flowers begin to secrete nectar two or three days before reaching their full size, it was felt that assaying the flowers at maturity may have obscured the pattern of variation in nectar concentration. The concentration of freshly secreted nectar was found to be of the order of 10 to 20%; after three or four days exposure to the atmosphere, concentrations might be as high as 65%. A further study of

TABLE II

MEAN CONCENTRATION OF SNAPDRAGON NECTAR AT VARIOUS SOIL MOISTURE LEVELS—NECTAR WITHDRAWN PERIODICALLY.

Treatment	Number in sample	Mean concentration (%)
Moisture equivalent	26	20.0 ± 0.52
75–100% available water	27	19.9 ± 0.38
Moisture equivalent	34	17.2 ± 0.84
25–75% available water	47	18.1 ± 0.70
Moisture equivalent	17	17.1 ± 0.56
< 25% available water	10	20.4 ± 0.58**
Moisture equivalent	19	16.3 ± 0.47
Saturation—plants not wilted	19	16.8 ± 0.47
Saturation—plants wilted	10	34.1 ± 1.65**

*Standard error of a mean.
**Significant at 1% level.

variation in nectar concentration with soil water level was therefore undertaken. Instead of collecting the nectar when the flowers were mature, fine glass capillaries were inserted into the immature flowers in contact with the nectary tissue on the ovary surface. As the nectar was secreted it rose in the capillary tubes. Tubes were withdrawn twice daily for concentration readings and fresh ones inserted. In other respects experimental treatment was the same as in the previous experiment.

Nectar concentration comparisons between plants at moisture equivalent and plants in drying or saturated soil are given in table II. The general pattern is similar to that of table I, except that measured concentrations are much lower. Under conditions of extreme water deficit, whether in very dry or in waterlogged soil, sugar concentrations were relatively high.

Plants in a state of permanent wilt continued to secrete nectar as long as the flowers remained turgid. Secretion ceased in each flower as it wilted. Restoration of turgor to plants in soil at the permanent wilting percentage was followed by a resumption of secretion within one or two hours.

The effect of soil temperature on nectar secretion

Pre-experiment treatment of plants used in soil temperature studies was the same as in the soil moisture studies. When the lower flower buds were opening, 15 plants were randomized and placed in water baths held at 60°, 70°, and 80° ± 1° F. (15.5°, 21.1°, and 26.6° C.). Nectar concentration comparisons were made on centrifuged samples in order to use the maximal

TABLE III

MEAN DAILY YIELDS OF NECTAR SUGAR IN SNAPDRAGONS AT SOIL TEMPERATURES OF 60°, 70°, AND 80° F.

Date	Mean daily yield of nectar sugar (mg./flower)		
	60°	*70°*	*80°*
Jan. 31	3.01	3.88	3.36
Feb. 2	1.68	1.88	2.41
3	1.23	1.50	1.97
6	2.03	2.25	2.13
7	1.78	2.02	2.38
8	1.50	1.90	2.23
9	2.37	2.00	2.95
10	2.38	2.42	2.68

"F" values significant at the 1% level for the 80°/60° comparison, at the 5% level for the 70°/60° comparison, and non-significant for the 80°/60° comparison.

number of flowers for both concentration and volume measurements. Comparisons were based on the daily mean yields reported in table III. The 80° and 70° groups outyielded the 60° group. Differences between the 80° and 70° treatments fell short of statistical significance, though yields were generally higher at 80°. Volume yields closely paralleled sugar weight yields, while concentration differences were non-significant.

In a repetition of the experiment soil temperatures of 45° (7.2° C), 60°, and 75° were used. The yield pattern was similar to that shown in table III. Both the 75° and 60° groups outyielded the 45° plants, with "F" values significant at the 1% level. Differences between the 75° and 60° treatments fell short of significance, and in no instance were concentration differences statistically significant.

Although yield differences were not always clear-cut, it was apparent from the data of the two experiments that nectar yield was depressed at the lower soil temperatures, and that, as temperature was raised in the range between 45° and 80° F, there was a more or less linear increase in nectar production. Daily yield differences among the various treatments in both experimental series were uniform over the experimental period. It is unlikely, therefore, that they were a reflection of a progressive decline in plant vigor. No differences in flower size or number were apparent, nor were there any signs of even a temporary wilting in any treatment. There was, however, a slight chlorosis in the 60° plants and a marked chlorosis in the 45° plants, indicative possibly of a depression in nitrogen uptake or assimilation similar to that found in apple trees kept at low temperatures (**2, 5**).

The effect of a reduction in substrate aeration

The effect of curtailing substrate aeration was investigated in sub-irrigated sand cultures. Soil air samplers (**3**) were buried vertically about an inch below the sand surface in each plant container, and air samples were collected over acidulated water by suction. Oxygen and carbon dioxide contents were measured with a Haldane-Guthrie analyzer. When the first flower buds were opening, the sand surfaces in the treated series were sealed off with a ½-inch layer of Parowax, supplemented around the perimeter with a layer of heavy grease. In addition, the lower inch of sand in each container was kept saturated with the culture solution. In the control series there was unrestricted gas exchange between substrate and atmosphere. During the 8-day harvesting period composite air samples from each series were analyzed daily for oxygen and carbon dioxide. In the control group air composition remained essentially the same as in the atmosphere. In the treated group oxygen concentration gradually fell to 17%, then rose to 18.5%. The reason for the failure to obtain a more marked reduction in root aeration is not known. Plants in the sealed containers remained in excellent condition throughout the experiment, and no reduction in flower size or number was evident. Some 420 flowers were harvested in daily samplings beginning four days after treatment application. Nectar concentration comparisons were based on samples drawn directly from the flowers. Yield data were as follows: Control—mean volume 2.4 μl. per flower, sugar weight 1.7 mg. per flower, and concentration 62.3%; Treated—mean volume 2.1 μl. per flower, sugar weight 1.5 mg. per flower, and concentration 66.1%. These differences, while small, were consistent over the sampling period and were

highly significant statistically. No association was found between daily variations in nectar values and fluctuations in the composition of the air in the sealed containers.

Discussion

It is apparent from the data presented above that the physical state of the soil during the flowering period may influence both yield and sugar concentration of nectar. Deviations from the optimum were attended by reduced nectar yields, even when the experimental treatment had little adverse effect on vegetation and no visible effect on flower development. The low yields of clover nectar noted in clay soil in years of adequate rainfall and in sandy soil in drought years (**4**) may have been due in part to soil physical factors operative during the flowering period.

Under conditions of extreme soil water deficit or reduced aeration, volume and total sugar weight of nectar were reduced, while the ratio of sugar to water was apparently increased. Under conditions of low soil temperature, or in drying soil in which only temporary wilting occurred, volume and total nectar sugar decreased, but concentration remained relatively constant. The effect of the soil factors appears to have been complex, and the manner in which they acted is not clear. Daily yield differences in the temperature and aeration studies were consistent over the experimental period and would therefore not appear to have been the result of a progressive decline in plant vigor. In drying soil, a water stress in the plant may have been the immediate cause of the diminution in secretion. In the soil temperature and aeration experiments, however, there was no visible evidence of such a stress. In view of the wilting in plants in drying soil, and the chlorosis in plants kept at low soil temperatures, it is possible that reduced nectar flow was the result of a reduction in sugar supply. WYKES (**9**) has found for horse chestnut that restrictions on sugar supply to the flower caused by defoliation or ringing lowered nectar volume, total sugar, and concentration.

The data contain no basis for an elucidation of the secretion mechanism. The persistence of secretion in flowers of badly wilted plants, so long as the flowers remained turgid, would indicate that the process is not dependent on a hydrostatic pressure transmitted through the vascular system of the whole plant. There is no answer to the question of the degree of interdependence of secretion of the sugar and water components of nectar, however, and the reason for the secretion of relatively highly concentrated nectar under certain treatment conditions remains obscure.

Summary

The physical state of the soil during the flowering period was found to influence both yield and sugar concentration of nectar in snapdragons. In very dry or imperfectly aerated soil, yield was reduced and concentration increased; in drying soil of more than 25% available water and in cold soil yield was reduced but concentration was not affected.

Yield reductions may have been due in part to restrictions on the sugar supply to the flower. The cause of variation in nectar concentration is not apparent.

Secretion was observed to continue in severely wilted plants for as long as the flowers remained turgid.

The work reported here has been a part of the programs of the Legume Research Committee in Ontario and the Tree Fruit Research Committee. Thanks are due Professor G. F. Townsend, Apiculture Department, and various members of the Horticulture Department, O.A.C., Guelph, for their co-operation.

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THE EFFECT OF MAGNESIUM CONCENTRATION ON CHLOROPHYLL AND CATALASE DEVELOPMENT IN CHLORELLA

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Received September 16, 1952

Numerous studies, most notably those of VON EULER and his associates (**3**) and GRANICK (**8**), have suggested that the various plant metalloporphyrins are built up along common pathways of synthesis. Interest in the sequence of formation of chlorophyll and the other metalloporphyrin compounds in the plant cell has led to an investigation of the synthesis of chlorophyll and catalase in the green alga *Chlorella vulgaris* as affected by magnesium concentration. That catalase and chlorophyll synthesis are inter-related has been indicated by VON EULER and his associates (**3**) and several other authors (**2, 4, 13**) working with various types of chlorophyll-deficient plants. Chlorophyll is a magnesium porphyrin, while the prosthetic group of catalase is an iron porphyrin containing no magnesium. Magnesium deficiency might be expected to cause a differential effect on the formation of the two compounds, thus indicating their synthetic relationship in the plant and possibly elucidating to some extent the role of magnesium in the synthesis of porphyrin compounds.

The algal cells were cultured in nutrient solutions containing glucose and varying in initial magnesium concentration so that magnesium deficiency would occur at different stages of culture development. Different magnesium concentrations have been previously used by several groups of workers to limit the synthesis of chlorophyll by Chlorella cultures. SAIZEVA (**16**) presented data indicating that the magnesium content of the culture solution affects differently the process of chlorophyll formation and the growth of cells. More recent contributors (**6, 10, 11**) have been interested in magnesium deficient Chlorella primarily in order to observe the photosynthetic activity of cells varying in chlorophyll content. Similarly, chlorophyll variation due to magnesium deficiency has been studied in a few other green algae (**12, 15**). The work of PIRSON and associates (**15**) is of especial interest here since their results indicate a deficiency response of cell multiplication and chlorophyll content in Ankistrodesmus similar to that found by us in Chlorella. In the present experiments the daily changes in the cultures were closely followed in order to correlate the effects on the metalloporphyrins with the other physiological changes which occurred as magnesium deficiency developed. Data on chlorophyll content, catalase activity, cell multiplication and cellular characteristics were analyzed. The latter two effects have been reported in a previous paper (**5**).

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The very large changes in cell size previously reported (**5**) made it difficult to find a suitable basis of comparison for the chlorophyll and catalase data. Only by correlating the findings on the basis of at least two different bases (*e.g.*, per cell and per unit volume of cells) could a picture of the changes taking place be composed.

Experimental procedures

The *Chlorella vulgaris* strain previously described was used, and the data of the present paper were derived from the same flask cultures, in fact from the same periodic samplings, as were reported in the preceding paper (**5**). The culture media contained glucose, and magnesium was present in the concentrations 0.2, 0.5, 1.0, 2.8, and 49 p.p.m., in replicate. The samples were treated as has been specified for the determination of population density and of volume of packed cells.

Chlorophyll was extracted from the cells by hot methanol as described by FLEISCHER (**6**). The method of extraction has been tested by HASKIN (**9**) and rechecked in our laboratory, where an insignificant amount of change in the chlorophyll extinction coefficient from the short exposure to hot methanol was found. The optical density of the extracts was measured at 6650 Å on a Beckman Model DU spectrophotometer. Although only relative values of chlorophyll concentration are required for a comparison of the chlorophyll content of the cells, an absolute value of chlorophyll concentration was obtained in order to simplify the comparisons. A solution of purified Chlorella chlorophyll which had been prepared by the method of WILLSTÄTTER and STOLL (**18**) was used as a standard. The extinction coefficient of this solution was found to be $k = 45$, when chlorophyll concentration is expressed as grams per liter.

Catalase activity was determined manometrically with a suspension of whole cells, in 1.0 *N* hydrogen peroxide at pH 7.0. (Attempts to break the cells by repeated freezing and thawing, or by grinding, produced no increase in catalase activity over that of the untreated cells.) The catalase method of APPLEMAN (**1**) was followed. After determination of the volume of packed cells (usually 0.1 to .2 ml.) the cells were quantitatively transferred to one arm of a Y-shaped catalase tube, using 0.1 M pH 7.0 phosphate buffer solution. Hydrogen peroxide in 0.1 M phosphate buffer solution, pH 7.0, was added to the other arm. (It was observed that 30% hydrogen peroxide (Baker's, Merck, or Eimer and Amend) when neutralized with KOH (or NaOH) and then mixed with pH 7 phosphate buffer resulted in an acid pH value. If the peroxide is first mixed with phosphate buffer and then neutralized with alkali, no pH difficulty is encountered on mixing the solution with additional buffer solution.) After cooling to 0.0° C, the solutions were mixed and the oxygen evolved was measured in a constant pressure manometer over a period of a few minutes, with continuous shaking. A graph of oxygen evolved plotted against time gave a sigmoid curve (**1**). The rate of oxygen evolution was defined as the maximum slope

of this curve. This rate was found to be highly reproducible and linearly proportional to the amount of Chlorella cell material over a larger range of added material (0.05 to 0.40 ml. packed cells) than was required.

In this paper, catalase is discussed in terms of its activity rather than its concentration or amount, since activity is the property measured, and no data are available by which to judge whether activity and amount are proportional.

Experimental results

Chlorophyll

The growth of the Chlorella cultures was accompanied by a rapid rise in their chlorophyll content; then an abrupt cessation of chlorophyll synthesis took place (fig. 1). The level of magnesium in the original medium determined the degree of chlorophyll development. The curves of the figure, expressed as chlorophyll per unit volume of culture fluid, are similar in shape

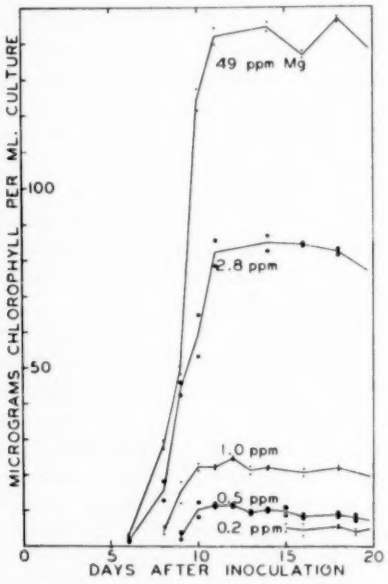

FIG. 1. Chlorophyll changes per unit volume of culture. Solid lines represent the averaged values from replicate cultures. Dots represent data from individual cultures.

to the curves of Chlorella population growth under the same conditions (5). In both sets of curves, the sharp leveling off in the lower-magnesium cultures at approximately day 10 is probably due to the same cause—depletion of magnesium, resulting in cessation of the reactions of chlorophyll formation and cell multiplication at this time. Both of these processes showed a vigorous revival when magnesium was added to the cultures (illus-

trated in (5) for cell population). It should be stressed, however, that at the time when magnesium became limiting to chlorophyll synthesis, and to population increase, the synthesis of cell substance was still progressing at a rapid rate in these cultures (5).

The data presentation of this paper is based primarily upon unit volume of culture fluid for the following reasons: a) it is an absolute basis, indicating the changes taking place in only one variable, so that (b) the variations in any culture characteristic may be compared, on an absolute basis, with other culture changes occurring at the same time. Other bases of comparison which might have been used do not have these advantages, as

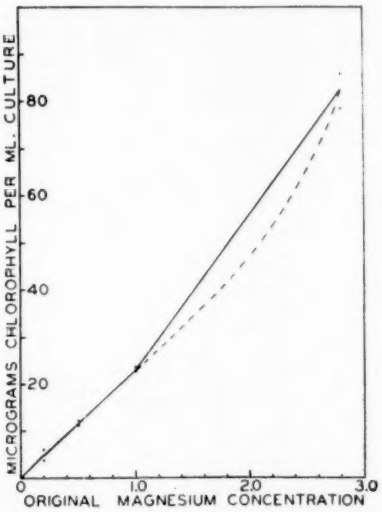

FIG. 2. Maximum chlorophyll attained by cultures of different initial magnesium content. Data are based upon determinations made on day 11 or the first sampling date thereafter. Solid lines represent the averaged values from replicate cultures and dots represent data from individual cultures.

will be seen later. It may be illuminating at this time, however, to notice the changes in chlorophyll per unit of cell substance. In figure 6, there is an initial rise in chlorophyll per unit volume of packed cells. In the low-magnesium curves this rise is followed by a rapid decline. The decline is not, however, due to any decrease in chlorophyll content in the cultures, as might at first appear. It is caused solely by the continued increase in cell substance (5) at a time when chlorophyll synthesis had come to a standstill and the culture chlorophyll content had leveled off. The latter fact was best established by the data on chlorophyll per unit volume of culture (fig. 1), as has been indicated.

The role of magnesium as the limiting factor for the synthesis of chlorophyll in the lower-magnesium cultures is more explicitly indicated in figure

2. In the media up to 2.8 p.p.m. Mg, the chlorophyll content of the cultures was almost proportional to the original magnesium concentration. The chlorophyll content attained by the cells represented approximately 70 to 80% of the total magnesium which had been added to the media. In contrast, the magnesium in the full-magnesium media (49 p.p.m.) was, in these cultures, probably not the factor which limited chlorophyll synthesis. By referring to figure 1, it can be seen that a 17.5-fold increase in magnesium concentration between the 2.8 p.p.m. and the 49 p.p.m. Mg cultures produced only a comparatively small (less than two-fold) increase in maximum chlorophyll content. Also, the later addition of 10 p.p.m. Mg to the

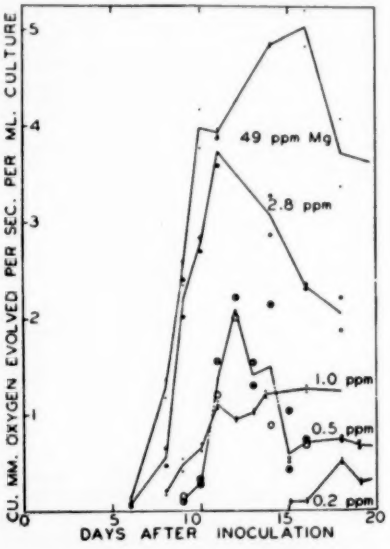

FIG. 3. Catalase activity changes per unit volume of culture. Solid lines represent the averaged values from replicate cultures and dots represent data from individual cultures. For the 0.5 p.p.m. Mg curve, the individual data from replicate cultures are singly indicated by using a different type of circle for each replicate.

49 p.p.m. cultures had no effect on their development. Limiting conditions other than magnesium concentration (probably nitrogen deficiency) are clearly indicated in the 49 p.p.m. cultures.

CATALASE

Changes in culture catalase activity (fig. 3) followed those of chlorophyll formation in three respects. (a) There was an initial increase in catalase activity per unit culture volume at each level of magnesium concentration, followed by a leveling off, or decline, in catalase activity. (b) The final levels of catalase activity varied according to the original magnesium concentrations in the respective cultures. (c) The addition of more

magnesium to the low-magnesium cultures after their catalase activities had leveled off produced renewed increases in catalase activity (not shown in the figure). In one respect, however, the catalase curves differ greatly from those of chlorophyll. On approximately day 10 at the time when chlorophyll synthesis (and cell multiplication) ceased in the cells grown in 0.5 p.p.m. Mg media, there was an unusually large, transient increase in catalase activity in these cells. This increase took place rapidly, over a period of only two days; then catalase activity dropped to a low level, where it remained. The curves for 1.0 p.p.m. Mg and greater do not show this increase in catalase activity after chlorophyll synthesis had ceased.

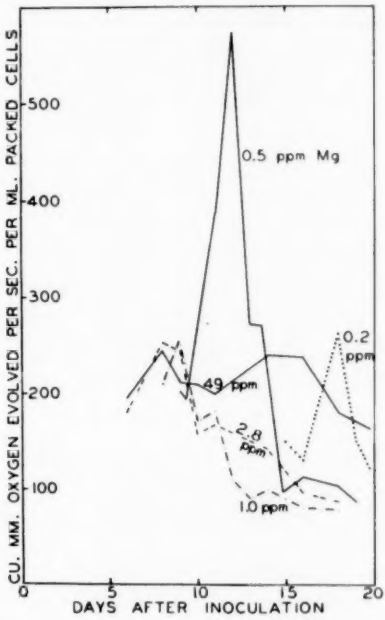

FIG. 4. Catalase activity changes per unit volume of packed cells. Averaged values from replicate cultures.

The magnitude of the large increase in catalase activity in the 0.5 p.p.m. Mg cultures is brought out more clearly in a curve based on catalase activity per volume of packed cells (fig. 4). The peak in catalase activity was three times as great as the maximum activity of any of the other cultures at any time in their development. In the 0.5 p.p.m. Mg cultures, catalase activity per cell (not illustrated) also showed this increase, and in even greater degree. On day 12 the average catalase activity per cell of these cultures was four times as high as it had been on day 10. Seven days later (day 19) it was back to approximately the same level as on day 10.

Whether or not the 0.2 p.p.m. Mg cultures followed this course is not

known, since the sampling of these poorly growing cultures was begun considerably later. But, experiments preliminary to the present sets of cultures had shown the effect for cultures initially containing 0.3 and 0.6 p.pm. Mg and, as in the later experiment, not for 1.0 p.p.m. Mg or over, as is briefly indicated in table I. In the 0.3 and 0.6 p.p.m. Mg cultures, catalase activity rose from approximately 200 to over 600 catalase rate units per

TABLE I

CATALASE ACTIVITY DATA FROM A PRELIMINARY EXPERIMENT. CU. MM. O_2 EVOLVED PER SEC. PER ML. PACKED CELLS. AVERAGED VALUES FROM REPLICATE CULTURES. ANALYTICAL AND CULTURAL METHODS SAME AS DESCRIBED.

Days after inoculation	Mg in the medium*		
	0.3 p.p.m.	0.6 p.p.m.	1.0 p.p.m.
10	.....	165	162
13	297	728	143
15	632	243	224

*Data from cultures containing 2.8 and 49 p.p.m. Mg not shown.

milliliter packed cells in two days (compare fig. 4), then rapidly dropped off in activity, just as had the already-discussed 0.5 p.p.m. Mg samples of the more recent experiment. APPLEMAN (**2**) has presented data which show a high catalase activity, as compared to the controls, during the early growth of the first leaf of low-magnesium barley seedlings, which activity then declined sharply in a period of a few days.

Discussion

After a period of growth, the various concentrations of magnesium in the different culture solutions had decreased, and the lower-magnesium cultures displayed signs of magnesium deficiency. This deficiency was manifested in the following ways:

(a) Cessation of cell division, (b) enlargement of the cells, (c) cessation of chlorophyll synthesis, and (d) effects on catalase activity. The catalase effects were, in the 1.0 and 2.8 p.p.m. Mg cultures, cessation of increase in catalase activity and in the lower-magnesium cultures, with a large rise in catalase activity followed by a decline two to three days later and no further increase. The 0.5 (0.3 and 0.6) p.p.m. Mg cultures showed the latter effect clearly. The first three effects and the effect on catalase activity in the 1.0 and 2.8 p.p.m. Mg cultures appeared simultaneously and seem definitely the result of magnesium unavailability. They could be reversed by addition of more magnesium to the cultures. The rise in catalase activity in the low-magnesium cultures appears also to have been a result of magnesium depletion since it occurred at the same time as the other symptoms, was quite transient, and, quickly falling to a low level of catalase activity, gave way to an apparent cessation of catalase synthesis.

These data suggest a piling up of the iron-porphyrin compound catalase when the synthesis of chlorophyll (or, its magnesium-porphyrin precursors) is blocked by the unavailability of magnesium for magnesium-porphyrin synthesis. The catalase iron-porphyrin may be in the direct line of chlorophyll synthesis (involving the removal of the iron atom and its replacement by magnesium); or, as seems more likely, catalase and chlorophyll may be derived from a common porphyrin precursor. According to the latter view, the unavailability of magnesium for magnesium-porphyrin synthesis leaves more protoporphyrin available for the synthesis of catalase iron-porphyrin. An observation by GILBERT (**7**) that the iron-porphyrin enzyme peroxidase displays increased activity in magnesium-deficient tung leaves is pertinent here, since the prosthetic groups of catalase and of peroxidase are very closely related or possibly identical (**17**).

Two questions require consideration. After the great increase in catalase activity accompanying magnesium-depletion in the 0.5 p.p.m. Mg cultures, what caused the subsequent cessation of increase in catalase activity? Why did a similar large increase in catalase activity following partial magnesium depletion not manifest itself in those higher-magnesium cultures which also displayed magnesium-deficiency symptoms? The first question may be related to the requirement for magnesium, at the enzymatic level, for porphyrin synthesis. A level of magnesium which would be insufficient to furnish the large amounts of magnesium required for chlorophyll synthesis might still maintain the enzymatic reaction of porphyrin synthesis. Further depletion of magnesium by the cells might halt the porphyrin-synthesizing reactions as well. The second question, concerning the absence of a large increase in catalase activity when magnesium became deficient in the higher-magnesium cultures, may be simply a matter of relative rates. In the 0.5 p.p.m. Mg cultures, of relatively low population-density, the time interval between the cessation of chlorophyll synthesis and the apparent cessation of catalase synthesis was two to three days. In the cultures with 1.0 and 2.8 p.p.m. Mg, the much denser populations would utilize the available magnesium much more quickly. If the cessation of chlorophyll synthesis takes place at a certain low level of magnesium concentration in all cultures, then, while the 0.5 p.p.m. Mg cultures required two or three days to further reduce this concentration to the level where it was unavailable for porphyrin synthesis, the higher-magnesium cultures with their higher cell populations would do this in much less time. Any rise in catalase might be missed, or it might not occur at all. These two questions can only be discussed speculatively since no quantitative data are available as to the magnesium concentration levels at which these syntheses become limited.

It is suggested, from the sequence in which the production of the respective materials ceased, that different minimum levels of magnesium concentration are required by the various synthetic reactions in question, and that the minimum culture-solution levels of magnesium concentration required

for these syntheses to take place are in the following order: Relatively high Mg, but < 0.2 p.p.m. (10^{-5} M) is required for chlorophyll synthesis and cell division, less is required for catalase synthesis (porphyrin synthesis?), and still less is required for synthesis of protein and other cell material contributing to cell enlargement.

This suggested sequence is based only on the data presented here and previously (**5**), which are subject to the following limitations: (a) Catalase activity is measured, not its content. Changes in activity may not accurately reflect increases and decreases in the catalase iron-porphyrin content. (b) The cell enlargement noted in the low-magnesium cultures after the onset of magnesium deficiency may represent, rather, lack of separation of newly formed cells (**5**). This being so, not cessation of cell division but,

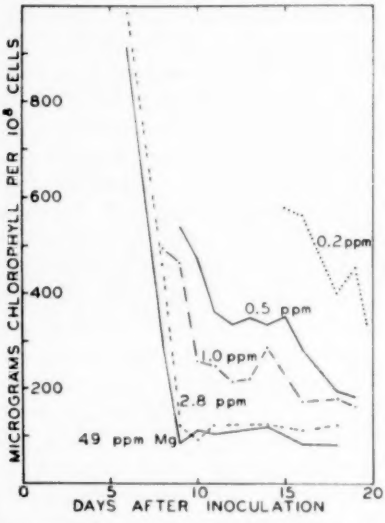

FIG. 5. Chlorophyll changes per 10^8 cells. Averaged values from replicate cultures.

instead, lack of cell separation would then be one of the first symptoms of magnesium deficiency. To our knowledge, no definitive cytological study of this phenomenon is available.

A difficulty encountered in the interpretation of results from algal cultures of this type is illustrated in figures 5 and 6. The graphs in both figures represent the same chlorophyll data; one figure is based on cell number, the other on the volume of packed cells, which is proportional to cell dry weight, or nitrogen content (**5**). The marked differences shown between the two bases are due to the differential changes taking place between the rates of cell division and the rates of syntheses in the cultures. The initial very rapid decrease in chlorophyll per cell observed in figure 5 (even while rapid chlorophyll synthesis was taking place in the cultures)

is accounted for by the decrease in cell size which occurred during the early period of active cell division (**5**). In figure 6, showing changes in chlorophyll per unit volume of packed cells, the abrupt reversals in slope in the lower-magnesium curves are chiefly due to the initially rapid rate and subsequently restricted rate of chlorophyll synthesis compared to the continuing rate of synthesis of gross cell material (**5**). During the initial few days (days 6 to 10), while there was a marked decrease in chlorophyll per cell, there was at the same time an increase in chlorophyll per unit volume of packed cells for all of the sampled cultures. At first glance, if the curves were studied independently, figure 5 would indicate a decline in

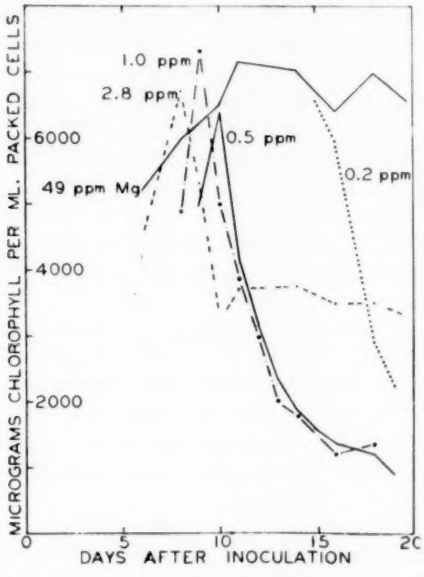

FIG. 6. Chlorophyll changes per unit volume of packed cells. Averaged values from replicate cultures.

chlorophyll and figure 6, an initial increase in chlorophyll, for data taken from the very same samples. Interpretation of either of these curves alone might easily give misleading results.

Such rapid changes in cell size and in the relative rates of syntheses of cell constituents may be responsible for discrepancies in results appearing in the literature, where the authors evaluate their results on only one basis (e.g., nitrogen content, or cell volume, or cell count) without regard to other changes occurring in the algal culture. It is also to be noted that in figures 5 and 6 a difference of one day in sampling date may make a tremendous difference in the numerical values of the data obtained. Which of the many possible bases of measurement best represents growth has been

asked in a similar connection by PEARSALL and LOOSE (**14**). By using several bases for his analytical data, the experimenter may attempt to get an overall picture of the relative changes occurring in his cultures.

Summary

The effect of magnesium concentration on the syntheses of the metalloporphyrins chlorophyll and catalase in *Chlorella vulgaris* was investigated. The cells were cultured in nutrient solutions containing glucose and differing in magnesium concentration so that magnesium-deficiency were produced at the lower concentrations. Changes in chlorophyll content, catalase activity, cell number and cell characteristics were analyzed.

Symptoms of magnesium deficiency appeared in the lower-magnesium cultures after about 10 days of growth. The following effects of the deficiency were observed on the metalloporphyrins studied. (a) cessation of chlorophyll synthesis, the chlorophyll content of the cultures being almost proportional to the original magnesium concentration of the medium in the low-magnesium and intermediate cultures (0.2 to 2.8 p.p.m. Mg), (b) cessation of increase in catalase activity in the intermediate-magnesium cultures (1.0 to 2.8 p.p.m. Mg), and (c) a transient large rise in catalase activity, followed by a decline, in the low-magnesium cultures. At the time when these deficiency-effects occurred, cell material was still being synthesized at a rapid rate.

These findings indicate that magnesium is required for both chlorophyll and catalase synthesis. The large rise in catalase activity concurrent with the cessation of chlorophyll synthesis, which occurs in some cultures, is interpreted to indicate that catalase (or porphyrin) synthesis does not require as high a concentration of magnesium as does chlorophyll synthesis, and that there exists a close biosynthetic relationship of the two substances during their formation in the plant.

A difficulty in the interpretation of results is caused by large changes in the algal cell size. Some aspects of this difficulty are given in example, and suggestions are made by which it may be overcome.

The authors wish to express their appreciation of the assistance of Mr. P. M. Shugarman in the analytical work.

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THE EFFECT OF MAGNESIUM CONCENTRATION ON GROWTH OF CHLORELLA

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Received September 16, 1952

A study of chlorophyll synthesis in *Chlorella vulgaris* (**3**) revealed striking differences in the growth patterns of this alga when cultured in nutrient solutions containing different amounts of magnesium. The differences in growth patterns were so variable and of such magnitude as to make difficult the finding of a suitable basis of comparison for physiological studies. Since Chlorella species are often the subject of such studies, a more exact knowledge of their growth habits seems desirable. The detailed studies of Chlorella made by PEARSALL and LOOSE (**7**) and MYERS (**6**) and numerous papers contributed by other workers have done much to advance the understanding of algal growth under various modifications of the culture conditions. This paper limits itself to comparisons of Chlorella growth at different magnesium concentrations. Data on certain aspects of this problem have been previously reported by SAIZEVA (**9**) and by VAN HILLE (**5**).

Materials and methods

Conical 500-ml. Pyrex flasks with sealed-in aeration tubes were autoclaved. Each flask was then filled with 350 ml. of sterile culture medium from a transfer flask in which as much as three liters of medium could be autoclaved in one batch (**2**). The full nutrient solution was prepared with reagent grade chemicals and contained the following components per liter of solution: KNO_3, 10 m.mol.; $Ca(NO_3)_2$, 3 m.mol.; $MgSO_4$, 2 m.mol.; KH_2PO_4, 2 m.mol.; ferric tartrate, 1 ml. of 0.5% solution; the micronutrients B, Mn, Zn, Cu, and Mo, in the amounts used by ARNON (**1**); glucose, 10 gm.; and Pyrex-redistilled water, to volume. The pH value of the autoclaved solution was 4 to 4.5. This solution was found to give consistently high culture densities, of the order of 200,000 cells per cu. mm. after a growth period of three weeks. In the media with lower magnesium contents, equivalent amounts of K_2SO_4 were substituted for the deficit in $MgSO_4$. Glucose was supplied in the media in order to maintain a source of sugar for all cultures, whether or not they developed a chlorosis, and hence minimize the influence of starvation effects on cell growth.

The magnesium concentrations used in these experiments were: 49 p.p.m. (full-magnesium), 2.8, 1.0, 0.5, and 0.2 p.p.m. Triplicate cultures of the first three concentrations were made; the latter two concentrations were run in

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sextuplicate, partly to expedite sampling of these poorly growing cultures. In media to which no magnesium had been added, no growth was discernible.

The strain of *Chlorella vulgaris* was obtained from Dr. Robert Emerson in 1931 and has been subcultured in this laboratory since that time. These subcultures are characterized by extremely poor growth in the absence of light (**4**), or when calcium is not added to the solution. Very dilute suspensions of cells taken from nutrient agar slants were pipetted into culture flasks, to give initial concentrations of approximately one cell per cubic millimeter of medium. The cultures were continuously aerated with air containing 5% carbon dioxide and saturated with water by bubbling through a water scrubber. The culture flasks were uniformly illuminated with 100 foot-candles of light from a set of small tungsten-filament bulbs at a distance of approximately 20 cm. below the flasks. The flasks were continuously shaken to keep the cells in suspension. Twenty-four 500-ml. flasks was the capacity of the shaking apparatus, described elsewhere (**2**). A temperature of 25 ± 0.5° C was maintained in a constant temperature room.

A difficulty arose which has also been experienced by other investigators interested in the cultivation of algae. The pH of the culture medium tended to rise gradually and after 10 to 20 days was in the range pH 7 to 8. Attempts to control this shift in pH by modifying the composition of the culture medium were unsuccessful. As a control on the possible effects of pH changes on the cultures, small amounts of sterile dilute sulphuric acid were added aseptically to several of the flasks after they had reached a high pH value, and the results were observed.

For the periodic determination of nitrogen content and dry weight during growth, much larger cultures were employed. Flasks with a capacity of 4 liters, identical in construction with the smaller flasks and containing 2500 ml. of culture medium, were inoculated with the same initial cell concentration as was used in the smaller flasks. Two concentrations of magnesium, 0.5 and 49 p.p.m., were used in single cultures. The flasks were maintained at room temperature (20 to 25° C, summer) in a well-lighted northern window, with moist air continuously bubbling through the cultures. They were shaken only a few times each day. Although this experimental treatment was not optimum, it is believed that for the specific purpose contemplated, the developmental changes in these cultures, though slower, followed adequately the growth patterns of the smaller cultures.

The volume of cells in samples periodically withdrawn from the cultures was determined after suspending the cells in M/10 pH 7 phosphate buffer and centrifuging for five minutes at 1300 × g. in tubes graduated in 0.01-ml. divisions (**10**). Repeated centrifugations performed in a uniform manner, or the comparison of packed volume with the chlorophyll content of the cells, gave results in agreement within ± 3%. Population density of the culture was determined by cell counts, in a standard hemocytometer. Duplicate counts of at least 250 cells each were made. Dry weight was determined after repeated drying at 95° C. The amount of nitrogen in the cells was determined by a semi-micro Kjeldahl procedure.

Sampling of the cultures was initiated as soon as sufficient growth had taken place to make analyses feasible. The sampling was performed in such a manner that, during the periods of rapid growth, two cultures of each treatment were sampled each day. Of the triplicate cultures, two were sampled one day, one of these and the third were sampled the next day, etc., so that each culture was sampled twice in three days. In the case of the sextuplicate cultures, three of these (a, b, and c) were analyzed in the same sequence as above, until one flask of these was empty, then another of the replicates (d) took the place of the emptied culture flask in the sampling sequence. This procedure ensured a continuous picture of the changes in each culture, coupled with replication and the conservation of culture material. The results obtained from the replicate cultures were usually quite close, such that a curve representing the averaged values had the same general shape and magnitude as curves representing the individual replicates. In the figures which follow, data from individual cultures, rather than the averaged data of replicates, are presented since it was thought desirable to indicate the effects of later magnesium additions upon the development of individual cultures.

Experimental results

The differences in cell population produced by variations in culture magnesium are shown in figure 1. It is particularly to be noted that after a rapid rise in cell number, the populations in the cultures with lower concentrations of magnesium leveled off. Cell multiplication was interfered with, presumably because of the depletion of available magnesium. In cultures containing up to 1.0 p.p.m. added magnesium, the average cell count after the rapid rise was proportional to magnesium concentration. Indications of a similar effect of magnesium deficiency on the growth of the alga Ankistrodesmus are seen in the recent report of Pirson *et al.* (**8**). It is interesting that in the present studies, the cultures of different initial magnesium concentrations, with the exception of the full-magnesium cultures, stopped rapid multiplication at approximately the same time (on approximately the tenth day). Apparently the higher magnesium concentrations induced more rapid cell division, which used magnesium at such an increased rate as to deplete magnesium in the various cultures at about the same time.

The introduction of an additional 10 p.p.m. of Mg, indicated by the arrows in the figure, was made to some of the flasks in order to test whether magnesium depletion was the limiting factor in cell multiplication. That this was so is shown conclusively in the lower-magnesium curves. The addition of small amounts of sterile acid to replicates of these cultures in order to bring the culture pH down to approximately pH 6 produced no changes in the population curves, hence eliminating pH as a limiting factor in these experiments.

The curves showing volume of packed cells per unit of culture fluid (fig. 2) also show dependence of this measure of growth upon the magnesium

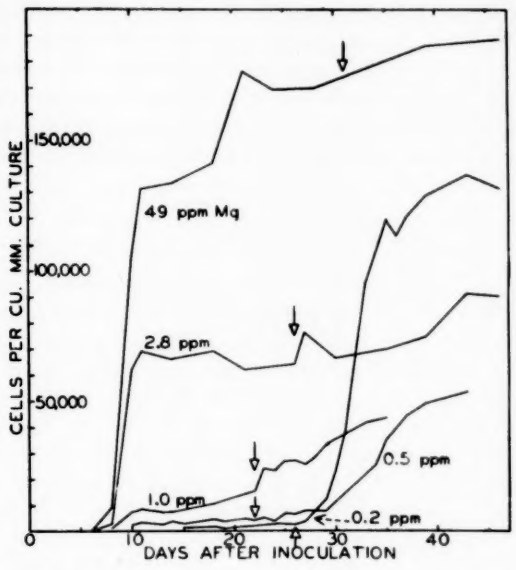

FIG. 1. Population density at different initial magnesium concentrations. Arrows indicate the addition of 10 p.p.m. Mg to the cultures. A single set of culture data is represented.

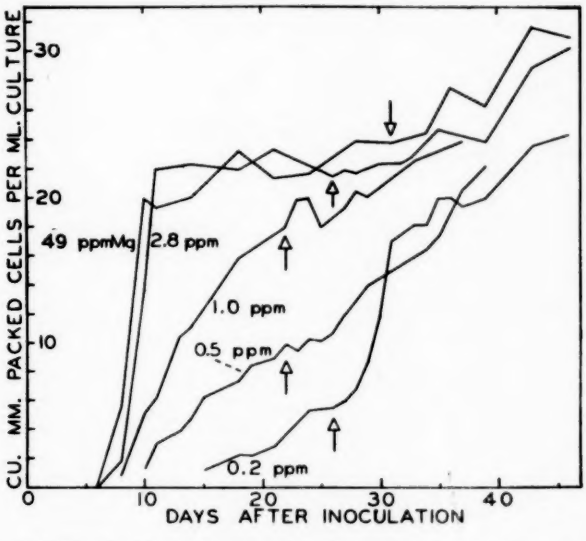

FIG. 2. Volume of packed cells per unit volume of culture at different initial magnesium concentrations. Arrows indicate the addition of 10 p.p.m. Mg to the cultures. A single set of culture data is represented.

concentration of the medium. But there is one striking difference between these curves and those of cell population in the lower-magnesium cultures, cell multiplication ceased at the onset of magnesium deficiency, while the volume of packed cells continued to increase at a rapid rate (cell size was increasing). The question as to what was happening to cell size before magnesium deficiency occurred was answered by making the calculations of algal size in the form

$$\frac{\text{Volume of packed cells/ml. culture}}{\text{Number of cells/ml. culture}}.$$

These calculations confirmed the increase in the size of cells under conditions of magnesium deficiency and in addition pointed out some other changes in cell size during the growth cycle (fig. 3). During the active growth of the first 10 days (starting with cells taken from agar slants) there was a rapid progressive reduction in size, as had been found in some degree in other laboratories (**7**). From the curves of figure 3 it can be seen that the size of the newly introduced cells was probably greater than 2000 cu. μ, the volume subsequently decreasing to as little as 100 cu. μ (average packed size)—

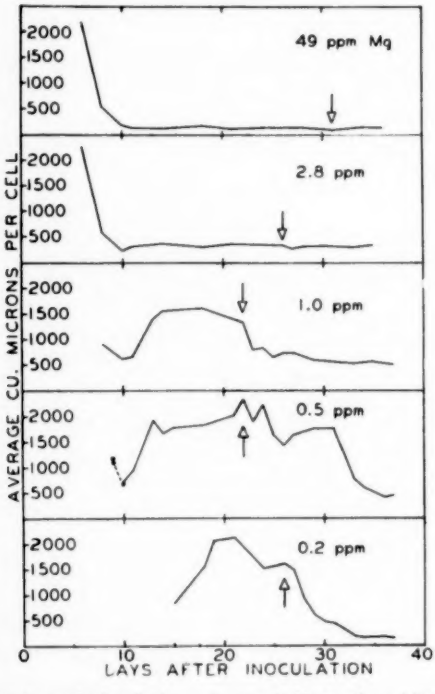

FIG. 3. Changes in packed cell size. Arrows indicate the addition of 10 p.p.m. Mg to the cultures. Solid line represents a single set of culture data. Circles represent data from other replicates of the cultures with 0.5 p.p.m. Mg.

a 20-fold change in cell volume. The data for the early portion of the graph do not appear for the cultures of lowest magnesium concentration because these slower-growing cultures were not yet sufficiently developed to permit sampling. But it is evident that these, too, must have gone through the initial decrease in cell size since all cultures were inoculated from the same cell suspension. The smallest cell size was attained under the highest magnesium concentration; and the small size was maintained in the high-magnesium cultures for the duration of the experiment. But in those cultures where magnesium was reduced to an unavailable level, at around the tenth

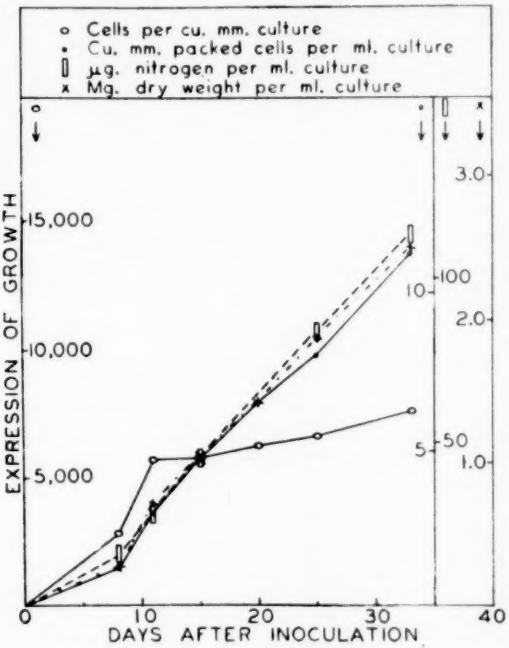

FIG. 4. Large-flask culture, 0.5 p.p.m. Mg. Growth expressed in various terms. Curves drawn so as to meet at fifteenth day.

day, an increase in cell size was initiated. The transition from decrease in cell size to cell enlargement took place concurrently with the cessation of cell multiplication. That these changes were effects of magnesium deficiency is again indicated by the fact that, in the low-magnesium cultures, additions of magnesium reversed the trend of change in cell size. Such tremendous variations in size make the value of expressing results on a per cell basis dubious unless great caution is exercised in the interpretation of the data, in which case valuable information may be obtained.

It should be noted that the cells used for inoculation, as seen from their size, were not at a vigorous growth stage at the time they were taken from

the agar slants for inoculation. That they attained a much more vigorous condition after a few days in the high-magnesium media is apparent from the graphs of cell size; and that they initially approached such a condition in the low-magnesium media is also indicated. No data are presented to show the effect of introducing vigorously growing (small) cells in the inoculating fluid, but it may be supposed that the same patterns, minus the initial size decrease, would have occurred.

The question arose whether an increase in cell material or merely a swelling of the cells due to water uptake accounted for the increase in cell size following the cessation of cell multiplication. Large samples were withdrawn periodically from the cultures of 4 liters volume described previously and the following analyses were performed: cell count, packed volume of cells, nitrogen content, and dry weight. The results of these analyses are presented in figure 4 for the culture with 0.5 p.p.m. Mg. Nitrogen content, dry weight, and packed volume of cells continued to increase proportionally for a considerable time after cell multiplication had essentially stopped. It is evident that a continued synthesis of cellular material (increase in dry matter) was taking place at this low level of magnesium concentration. It was equally evident that in cultures with adequate magnesium (49 p.p.m.), changes in cell size were due to changes in dry matter and not to changes in water content.

Discussion

Conclusions about two aspects of cell growth can be drawn from the results of these experiments: one relates to the effect of magnesium concentration upon cell multiplication; the other bears on its effect upon synthesis of cell material. Both of these processes taken together determine the observed large differences in cell size. Under conditions of adequate magnesium, cell division is rapid. When magnesium becomes unavailable reproduction apparently ceases, or slows down considerably, but can be induced again by addition of more magnesium. Magnesium as a controlling factor in the division of bacteria has been reported by Webb (11). On the other hand, the process of synthesis of cell material seems to be little affected by the decrease of magnesium concentration (within the limits tested) during growth. That is, after the tenth day, when magnesium became unavailable for cell multiplication, the lower-magnesium curves of volume of packed cells per unit volume of culture continue to rise at about the same rate as previously. The synthesized material was going largely into the increase in cell size rather than into new cells. This continued synthesis of cell material was apparent for more than 10 days, after which the experimental conditions were changed by additions made to the cultures.

Clearly, these data indicate that the synthesis of cell material can take place at a lower concentration of magnesium in the culture than is required for cell multiplication. In fact, as appears in figure 2, the cultures with 2.8 p.p.m. and 1.0 p.p.m. of Mg attained approximately the same growth levels

in packed volume of cells as did the full-magnesium cultures. At this high level of growth (as appears from data obtained from the culture with 49 p.p.m. of Mg) nitrogen was probably limiting the further synthesis of cell material. Yet, although the accumulation of cell material in the cultures with 2.8 and 1.0 p.p.m. of Mg continued at a steady rate until factors other than magnesium became limiting in the media, the cell populations of these cultures were clearly restricted by magnesium (fig. 1), the cultures with 1.0 p.p.m. contained less than one tenth the cell number present in the cultures with 49 p.p.m. of Mg.

While the degree of magnesium deficiency attained in these experiments did not halt the production of cell material, magnesium did have a role in its synthesis. The slopes of the low-magnesium curves in figure 2 indicate a definite dependence of the rate of accumulation of cell material on the original concentration of magnesium in the culture medium. It is to be noted that the low-magnesium cultures were proportionately low in chlorophyll content (**3**). It is probable that this had an effect on the rate of photosynthesis. Yet it seems unlikely that, with M/20 glucose present in the medium, the observed decreases in the rates of accumulation of dry matter were due chiefly to lowered rates of photosynthesis.

It appears then that the process of cell division and the process of accumulation of cell material are limited by different metabolic factors requiring different levels of magnesium concentration. At the initial stages of culture development, with sufficient magnesium present, the rate of cell division was greater than the rate of synthesis of dry matter, giving a population of predominantly small cells. As the cultures developed and magnesium became deficient cell division stopped. Synthesis of cell material, being a process that can evidently proceed at lower levels of magnesium, continued at a rapid rate. It can readily be seen that in deficiency studies (and under other deleterious conditions) the apparent results of the study of Chlorella may vary greatly, depending on whether cell number or dry weight (similarly, packed volume and cell nitrogen) is used as a basis for comparison. Further investigation of the metabolic interactions controlling these two growth phenomena, cell multiplication and accumulation of cell material, would be of interest.

A complication arises in the interpretation in that the large Chlorella cells formed in the low-magnesium cultures were of an irregular form. Vigorously reproducing cells, produced when magnesium was readily available, were small and thin-walled, with visible chloroplasts. The large, non-dividing cells were opaque and apparently thick-walled, with markings of rectangular form visible on the walls. The interesting possibility that these cells were multicellular units and that magnesium deficiency caused, not the cessation of cell division but the hindrance of cell separation should not be overlooked. The observations of WEBB (**11, 12**) on bacteria may again be of interest here. The techniques at hand in the observation of this minute alga were not adequate to distinguish the two effects.

Summary

Comparisons were made of the growth patterns of *Chlorella vulgaris* when cultured in nutrient solutions containing glucose and differing in magnesium concentration. The amounts of magnesium used were such that symptoms of magnesium deficiency were produced at the lower concentrations. Growth was measured primarily in terms of increase in the cell population and increase in the volume of packed cells per unit volume of culture, the latter being an expression of the synthesis of cellular material.

In the full-magnesium cultures (49 p.p.m. Mg) cell population increased and the synthesis of cell material proceeded rapidly, being limited finally by conditions other than magnesium deficiency in the medium. The cells decreased greatly in size during the period of rapid growth and then remained at the same small size for the duration of the experiments.

Symptoms of magnesium deficiency appeared in the cultures initially containing 0.2, 0.5, and 1.0 p.p.m. Mg, while only some of these deficiency-effects were apparent in the cultures with 2.8 p.p.m. Mg. Magnesium deficiency interrupted cell multiplication at cell populations proportional to the original magnesium concentrations of the various media. The synthesis of cell material was not arrested hence cell size increased greatly, some of the cultures deficient in magnesium giving average cell sizes more than 20-fold larger in volume than the cultures with sufficient magnesium. These increases in cell size were paralleled by proportional increases in nitrogen content and dry weight.

It appears that cell multiplication and synthesis of cell material are independent processes in cultures of *Chlorella vulgaris* and that the process of multiplication requires a larger concentration of magnesium in the medium than does the production of cell material. Anatomical observations suggest that cell division (mitosis) was possibly taking place in the magnesium-deficient cultures but was not apparent owing to lack of cell separation. The limiting role of magnesium in the growth processes is discussed.

The authors wish to express their appreciation of the assistance of Mr. P. M. Shugarman in the analytical work.

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ALUMINUM TOXICITY STUDIES WITH RADIOACTIVE PHOSPHORUS

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Received September 25, 1952

The role of aluminum in the poor growth of certain plants on acid soil has been the subject of rather extensive investigations during the past fifty years. Investigators at the Rhode Island Agricultural Experiment Station suggested that low yields of certain crops grown on acid soils were due to the relatively high concentration of aluminum in those soils. An increase in yield as the result of liming was attributed in part to the reduction of the aluminum concentration in the soil solution. Heavy applications of acid phosphate gave even more striking results. At first this increase in yield after phosphorus application was also explained on the basis of the precipitation of aluminum in the soil. However, BURGESS and PEMBER (**2**) suggested that such mutual precipitation of aluminum and phosphorus occurred within the plant. When the heavy applications of acid phosphate were used, the corrective action was attributed to an excess of phosphorus being available for metabolic processes after some of the phosphorus had been inactivated internally by aluminum.

Although the majority of investigators who link aluminum with phosphorus as an explanation of aluminum toxicity suggest the precipitation of phosphorus by aluminum in the soil as the cause of the poor growth of plants, the data obtained by BURGESS and PEMBER (**2**), MCGEORGE (**5**), and PIERRE and STUART (**7**) in the course of their researches indicated to them that internal precipitation of phosphorus by aluminum may be important. These investigators did not mention any specific location where the internal precipitation took place. WRIGHT (**9, 10, 11**) presented evidence that internal precipitation is significant, and that deleterious effects are produced because of the inactivation of phosphorus by aluminum in the roots of the plants. A comprehensive survey and commentary on the literature prior to 1943 has been published by HUTCHINSON (**3**).

WALLIHAN (**8**) has reported that in his investigations he was unable to find evidence in support of the hypothesis that aluminum precipitated phosphorus within root tissues. Wallihan adopted a procedure reported in Wright's 1943 paper. Wright used a series of acids varying by 0.5 pH from pH 1.0 to pH 5.0 to extract phosphorus from dried and ground barley plants which were grown in culture solutions lacking aluminum, and from this same plant material to which known amounts of aluminum phosphate had been added. The acid at pH 3.0 just failed to extract the added aluminum phosphate, whereas the acid at pH 1.0 extracted all of the phosphorus in the

plant sample. Consequently, it was decided that these two acids could be used to distinguish between phosphorus bound by aluminum and phosphorus occurring in other forms. Therefore, these acids were used to extract the phosphorus from barley plants grown in culture solutions with and without aluminum, and the results were interpreted to indicate a precipitation of phosphorus by aluminum, primarily within roots.

Wallihan criticized this technique on the basis that extraction with these acids failed to distinguish between phosphorus on roots and that in roots, and concluded that the data could be explained solely by the presence of materials on the roots at the time of harvest. To check this point he grew Ladino clover in culture solutions with and without aluminum, and then immersed the live roots in extracting solutions of 0.001 *N* sulfuric acid for fifteen minutes followed by 0.1 *N* acid for fifteen minutes. The aluminum and phosphorus extracted by the 0.1 *N* acid were assumed to be on surfaces because (1) large amounts were extracted in the relatively short time of fifteen minutes, and (2) test plants whose roots were washed in 0.1 *N* acid and then returned to the culture solution grew well and revealed no visible injury to the roots, thus leading to the conclusion that the acid did not penetrate and bring about the removal of minerals from within the roots.

The conclusions of Wallihan obtained from his research, and his statement that a review of the literature failed to reveal evidence in support of the suggestion that phosphorus is inactivated internally by aluminum served as the stimulus for further investigations by the authors. In order to obtain more information concerning the absorption and translocation of phosphorus it was decided to use radioactive phosphorus in culture solutions, and by the use of radioautographs ascertain the distribution of P^{32} in plants grown in culture solutions with and without aluminum. In addition it was proposed to determine the distribution of aluminum by a modification of Heidenhain's iron hematoxylin process.

Experimental procedure and results

After germination of seeds of *Hordeum vulgare*, Wisconsin No. 38, on perforated discs in distilled water, one week old seedlings were transferred to complete culture solutions containing 10 p.p.m. of phosphorus. After a growing period of ten days one half of the plants were transferred to culture solutions containing 10 p.p.m. of aluminum from $Al_2(SO_4)_3 \cdot 18\,H_2O$. At the end of four weeks those plants grown in contact with aluminum showed the typical symptoms observed in previous experiments with barley. The tops were stunted, leaves contained yellowish streaks, and a reddish discoloration was evident at the stem base. Roots were few, much shortened with stubby tips, and were brownish and somewhat brittle.

At the end of this four weeks' growing period, culture solutions with and without aluminum were made such that they contained approximately 15 microcuries of P^{32} per liter. Plants were transferred to these and allowed to remain for four hours. The root systems of separate plants were then

washed for fifteen minutes in tap water, 0.001 *N* sulfuric acid, and 0.1 *N* sulfuric acid, and immediately dried in an herbarium press for one hour. The plants were taken to a dark room and each plant from the different washings was placed on a sheet of 8″ × 10″ Kodak No-Screen X-ray film, placed between sheets of herbarium paper and solid herbarium press boards, weighted, and allowed to remain in contact with the film for two hours. Films were then developed using FR Developer and FR Fixer. A Wratten Safelight, series one, was used in the darkroom.

Samples of roots which had been washed in tap water for fifteen minutes were fixed in Rollins-Carnoy F.A.A. No. 2 in preparation for dehydration and paraffin embedding.

The distribution of P^{32} in the barley plants is illustrated in figure 1. It can be seen by comparison of figure 1 A with figure 1 B that the top of the plant which was grown in a culture solution lacking aluminum contains con-

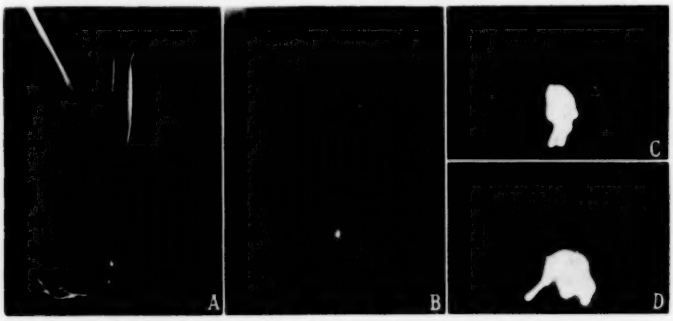

FIG. 1. Autoradiographs demonstrating distribution of radioactive phosphorus in barley plants grown in culture solutions without (A) and with (B, C, D) aluminum. A and B. Roots washed for 15 minutes in 0.1 *N* sulfuric acid. C. Roots washed for 15 minutes in tap water. D. Roots washed for 15 minutes in 0.001 *N* sulfuric acid.

siderable radioactive phosphorus whereas very little is evident in the top of the plant grown in contact with aluminum. These same results were obtained with all of the plants tested, both in preliminary experiments and the one here reported. This substantiates the hypothesis that phosphorus is largely inactivated on the root surface or within tissues of the root system by the presence of aluminum, and thus becomes unavailable for metabolic processes throughout the plant. Although these radioautographs cannot be used in a strict quantitative sense, certain conclusions can be drawn. To the eye there is not much difference between the amount of P^{32} in the aluminum-supplied roots which were washed for fifteen minutes in tap water (fig. 1 C) and those which were washed for fifteen minutes in 0.001 *N* acid (fig. 1 D), indicating not much difference in solubility of the phosphorus in these two solvents. However, much of the P^{32} was removed from the aluminum-supplied roots which were washed for fifteen minutes in 0.1 *N* acid (fig. 1 B). Since these two acids as extracting media can be used to distinguish between

phosphorus bound by aluminum and phosphorus not so bound, it is assumed that this differential amount of radioactive phosphorus remaining after extraction with these two acids is further evidence supporting the concept that the phosphorus is bound by aluminum in the root system.

These radioautographs do not differentiate between phosphorus precipitated on roots as distinct from that precipitated in roots. However, certain interpretations can be made. It is reasonable to assume that when the roots are placed for a short time in the extracting medium, surface phosphorus will first be removed, and then phosphorus will be removed progressively inwards until the end of the fifteen minute period terminates the process. Any phosphorus remaining at the end of this period will in all probability be within the root. Since there is phosphorus remaining in those roots, especially at the tips, which were extracted with 0.1 *N* acid (fig. 1 B), this phosphorus is evidently internally bound, internal for the reasons just cited, and bound because it does not reach the top of the plant in any appreciable quantity.

In a preliminary experiment approximately 5 microcuries of P^{32} per liter of culture solution were used, and the plants were placed in the solutions for two hours. After extracting separate root systems for fifteen minutes in tap water, 0.001 *N* acid, and 0.1 *N* acid, radioautographs were made. However, a blank negative was obtained for the plants whose roots had been washed in 0.1 *N* acid. In this particular instance all of the P^{32}, both on and in the roots, had been removed. This further strengthens the viewpoint that the remaining P^{32} in the 0.1 *N* acid washed roots from plants grown in higher concentrations of P^{32} for a longer period of time is internal. Wallihan's contention that the extracting acids did not penetrate the roots and, therefore, extracted only surface materials does not seem to be valid. It cannot be denied that precipitation occurs on root systems, but it also is evident that considerable phosphorus is inactivated internally.

Phosphorus has also been reported by Biddulph and Woodbridge (**1**) as being involved in precipitation reactions within the tissues of bean plants. They found that 0.00005 M P in the culture solution was sufficient for the continued growth of leaves. As more phosphorus was made available in the culture solution the phosphorus content of stems and petioles about doubled. This accumulation of phosphorus interfered with the passage of iron because of the precipitation of iron in the conductive tissues. The principal effect of high concentrations of phosphorus on the development of chlorosis was attributed to this immobilization of iron by phosphorus.

Transverse and longitudinal sections of roots were cut at 5 μ on a rotary microtome, and the ribbon was stretched and dried on slides at 40° C for at least one hour. In order to observe the presence and distribution of aluminum a procedure suggested by Johansen (**4**) was followed. This consisted in a modification of the usual staining process using Heidenhain's iron hematoxylin. Hematoxylin does not stain unless the plant material is mordanted, the most commonly used mordanting agent being ferric ammonium sulphate,

"iron alum." If tissues to be stained contain traces of aluminum, the dye will be taken up in those tissues where aluminum is present without iron alum treatment.

The distribution of aluminum in the roots grown in contact with aluminum is illustrated in figure 2, A and B. It will be noted that there is considerable stained material on the surface of the root, and that such stain is also found throughout the cortical region internally as far as the endodermis. The outer walls of the endodermis stained deeply, but the inner walls were practically free of stain. Very little stain, and hence very little aluminum, is found in the vascular system internal to the endodermis. Sections of roots not grown in contact with aluminum and treated with hematoxylin without a mordant were so lightly stained that satisfactory photomicrographs could not be obtained. These results indicate that aluminum

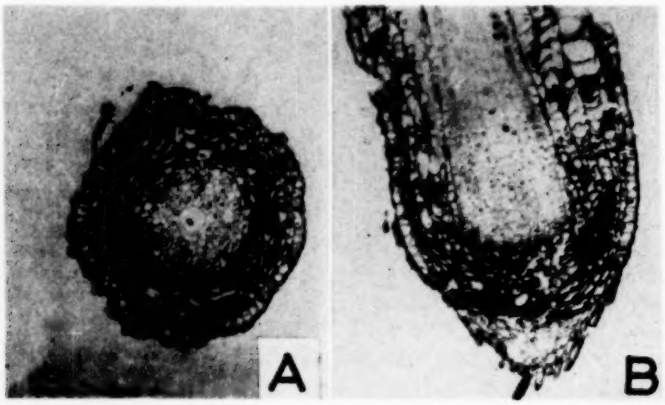

FIG. 2. Distribution of aluminum as indicated by dark stain in transverse, A, and longitudinal, B, sections of barley roots grown in culture solutions containing aluminum.

precipitation is not just a root surface phenomenon, but occurs within root tissues, a substantiation of similar results reported by MCLEAN and GILBERT (6). A cursory examination of the photomicrographs reveals considerable internal morphological variation from normal roots, further evidence that there is an upset of normal root metabolism.

These experiments concerning the distribution of radioactive phosphorus and of aluminum indicate that there is a mutual precipitation of these elements, that this condition is largely confined to the roots, and that, not discounting the occurrence of surface reactions, there is considerable inactivation of phosphorus by aluminum within the root.

Summary

Barley plants were grown in culture solutions with and without aluminum and then transferred to similar culture solutions to which approximately

15 mc. of P^{32} per liter of solution had been added. After four hours of exposure to the P^{32} root systems of separate plants were washed for fifteen minutes in tap water, 0.001 *N* sulfuric acid, and 0.1 *N* sulfuric acid, these acids being used to distinguish between phosphorus bound by aluminum and phosphorus not so bound. Plants were dried and pressed for one hour, and then exposed to Kodak No-Screen X-ray film for two hours. The radioautographs thus obtained demonstrated an apparently large accumulation of P^{32} in the root systems and very little P^{32} in the tops of those plants grown in contact with aluminum, results which contrasted sharply with the observed presence of considerable quantities of P^{32} in the tops of those plants not grown in contact with aluminum. Tap water and 0.001 *N* acid as extracting media behaved quite similarly, neither extracting any appreciable quantity of P^{32} from the roots. However, extraction with 0.1 *N* acid removed most of the P^{32}. This solubility in 0.1 *N* acid indicated that the phosphorus present was bound by aluminum, and that such inactivation of phosphorus by aluminum is in large part internal since any P^{32} remaining after a short period of time could not be phosphorus on root surfaces.

Cross and longitudinal sections of roots grown in contact with aluminum and washed for fifteen minutes in tap water were stained by Heidenhain's iron hematoxylin process modified to the extent that no mordant was used. Such modified procedure demonstrates the presence of aluminum wherever the stain is taken up. Aluminum accumulation was indicated on root surfaces and throughout the cortical region. Very little aluminum was present in the vascular system internal to the endodermis. The slides demonstrated that there was considerable modification of internal structure. This modification of plant structure and the distribution of aluminum and phosphorus as demonstrated in these experiments is further evidence in support of the general thesis that aluminum does inactivate phosphorus primarily within the roots of the plant and thus interferes with the normal phosphorus metabolism of the plant.

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FORMATION OF ESTERS BY YEAST. II. INVESTIGATIONS WITH CELLULAR SUSPENSIONS OF *HANSENULA ANOMALA*

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Received December 3, 1952

In a previous paper we reported (**21**) that *Hansenula anomala* produced ethyl acetate aerobically directly from ethanol. The present study with washed suspensions of cells was undertaken to determine optimal factors for ester formation and to obtain additional information on the mechanism for ester formation. While it was being concluded (**20**), PEEL (**18**) reported that ester formation from ethanol at pH 4.6 was greater in presence than in absence of 0.04 M acetate and investigated the effect of oxygen, age of culture, concentration of yeast cells, ethanol concentration, pH, and temperature on ester formation. Our results while in part confirmatory of those of Peel differ in several respects. This may be due to difference in strain used, or to the fact that our results were based on analysis of cellular suspensions incubated for longer periods of time.

Materials and methods

The strain of *H. anomala* var. *longa* type B (Naegeli) Dekker, was grown in medium I, TABACHNICK and JOSLYN (**21**) with 1% of glucose as carbon source. The medium inoculated with a 10% by volume of a 24-hour growing culture was shaken (7.5 cm. stroke, 94 cycles per minute) for 24 hours at room temperature (23 to 26° C). To prevent excess foaming, 0.3 ml. of sterile soy-bean oil was added per liter of shake culture. After harvesting the cells by centrifuging and washing, aseptic technique was no longer observed. Cell nitrogen was determined by the micro-Kjeldahl method.

Preliminary experiments showed that, as reported also by PEEL (**18**), ethanol alone could serve as substrate for ethyl acetate production and that acetate was not necessary. Acetic acid at a concentration of 0.05 M (at pH 3) was toxic. Highest yields of ethyl acetate were obtained in a 20-ml. cell suspension containing 10 mM of ethanol, 0.1 M phosphate buffer at pH of about 3, and at a cell density equivalent to 4.5 to 5.5 mg. of cell nitrogen per 20 ml., shaken in a cotton-stoppered 125-ml. Erlenmeyer flask for 18 to 24 hours at room temperature (23 to 26° C). Unless otherwise stated these conditions were used in subsequent experiments. PEEL (**18**) reported opti-

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mal yield of ethyl acetate from washed cells harvested from a 48-hour culture, pH 2.8 (McIlvaine citrate-phosphate buffer), 30° C, 9 mg. dry wt. cells/ml., 0.3 M ethanol and one hour of shaking in Krebs vessels containing a total volume of 10 ml.

At the completion of an experiment, the cells were centrifuged and saved for vital staining and microscopic examination. The initial and final pH were measured, and acetic acid, ethanol, and ester content were determined by methods described previously, TABACHNICK and JOSLYN (**21**). At first a control flask without yeast cells was shaken to determine loss of ethanol by evaporation but since this loss was never greater than 5% this was discontinued later.

Results

EFFECT OF SHAKING UNDER ANAEROBIC CONDITIONS.—No ester was formed with cell suspensions shaken under anaerobic conditions with ethanol alone or with ethanol and acetate at pH 2.1 or 6.7. Peel reported 5, 33, and 43 μmols of esters, respectively, in an 0.04 M acetate–0.1 M ethanol solution at pH 4.6 after shaking for one hour at 25° in nitrogen, air, and, oxygen, respectively. In confirmation of his results that ester formation is an aerobic process, we found under our conditions and with longer shaking that the addition of 0.5 mM acetaldehyde per 20 ml. also did not result in ester formation in the presence or absence of alcohol. Aerobically 3 mM of acetaldehyde were toxic; lower concentrations (0.5 mM to 1 mM per 20 ml.) although not toxic did not stimulate ester production. PEEL (**18**) found on the contrary that the addition of 0.01 M acetaldehyde at pH 2.8 (ethanol alone) or at pH 4.6 (ethanol + acetate) stimulated ester production, whereas at 0.2 M it inhibited ester production. Pyruvic acid (1–3 mM per 20 ml.) was respired aerobically and fermented anaerobically without formation of ester.

Autolysis of the yeast cells was not observed microscopically even when the majority of the cells in the microscope field appeared dead by vital stain.

OPTIMAL pH FOR ETHYL ACETATE PRODUCTION FROM ETHANOL.—The effect of pH on the accumulation of ester in 20 ml. of 0.1 M phosphate buffer containing 2.5 mM ethanol, and 1.25 mg. of yeast cell nitrogen per 20 ml. after incubation for 6 hours is shown in figure 1. Since the cells for our experiment were harvested at pH 2.4, it was possible that these cells had become adapted to ester production at a low pH. We therefore repeated the experiment and grew the cells in a medium containing 0.6% glucose and 0.05 M phosphate buffer at pH 6.7. These cells were harvested at pH 6.4. For yeast cells harvested at pH 2.4, the optimal pH was 2.1, and for those harvested at pH 6.4 it was 2.6. This optimal pH is the lowest reported for ester production by *H. anomala* (**9, 18**), but is not due to an adaptive mechanism resulting from harvesting the cells at pH 2.4. The cells harvested at pH 6.4 did produce more ester at the higher pH levels than those removed at pH 2.4 but the optimal yield of ester was obtained at pH 2.5. Since the optimal pH for cell multiplication of *H. anomala* is approximately pH 6, BEDFORD

(**2**), the accumulation of ethyl acetate at pH 2.1 to 2.6 apparently is related to a possible inhibition of the esterase at this low pH which resulted in increased accumulation of ethyl acetate in the medium.

In media with an initial pH of 6 or above, we usually observed 3 to 4 times more acetate than in those at lower pH (table I). Approximately the same amount of acetate was formed in 24 hours at pH 2.1 (if one includes the acetate present in the ester) as was formed in 43 hours at pH 6.7. Approximately the same amount of ethanol was utilized in both instances.

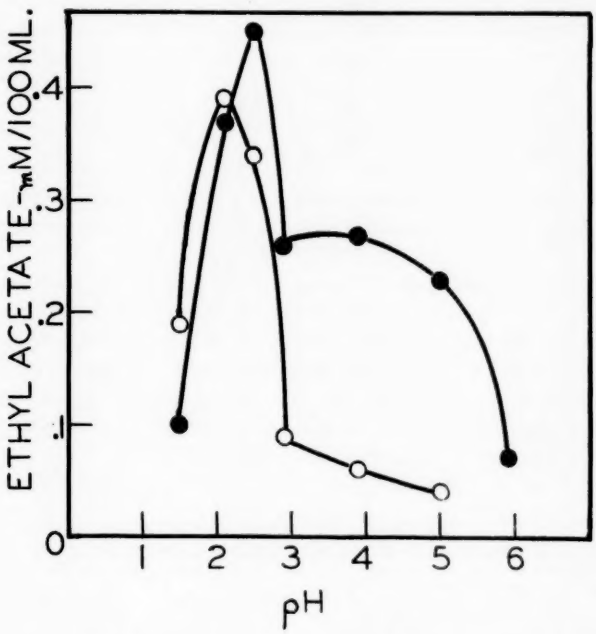

FIG. 1. Open circles = pH optimum curve, cells harvested at pH 2.4. Closed circles = pH optimum curve, cells harvested at pH 6.4.

EFFECT OF ETHANOL CONCENTRATION.—In preliminary experiments a range of ethanol concentration from 43 to 59 mM/100 ml. was found sufficient for optimal production and accumulation of ester under our experimental conditions. PEEL (**18**) reported optimal concentration of ethanol to be 0.2 M in presence or absence of acetate (pH 4.6 and 2.5, respectively).

As shown in table II, at pH 2.1, ethanol at concentrations of 100 mM/100 ml. or above (4.6% ethanol) appears to be toxic to *H. anomala*. At pH 6.7, ethanol at 186 mM/100 (8.7%) was toxic. The yields of ethyl acetate and acetic acid decreased at concentrations above these.

OPTIMAL TEMPERATURE.—The cell suspensions in separate one-liter rubber-stoppered flasks were stored in incubators at various temperatures. The

TABLE I

THE EFFECT OF SHAKING TIME AT pH 2.1 AND 6.7 ON ETHYL ACETATE PRODUCTION BY CELL SUSPENSIONS OF *H. Anomala.*

pH	Time in hours	Ethyl acetate mM/100 ml.	Ethanol remaining mM/100 ml.	Acetic Acid mM/100 ml.
2.1	6	1.57	47.1	1.43
	12	4.43	35.9	1.77
	18	6.20	26.1	1.14
	24	5.90	19.6	1.09
	35	4.20	14.9	.68
	48	1.90	10.9	.58
6.7	6	.24	48.4	1.09
	12	.16	45.5	1.35
	24	.05	35.7	2.38
	35	.02	24.0	4.90
	43	.04	19.3	6.95

flasks were shaken periodically during their 24-hour incubation period; in other respects the experimental conditions were the same as those described above.

The optimal temperature for ethyl acetate production was found to be about 19° C. At room temperature (23 to 26° C) the yield of ester was approximately 10% less. Vital staining showed 95 to 100% of cells to be dead, shrunken and granular after 24 hours at 32° C or above at pH 2.1.

EFFECT OF ACETIC ACID.—Acetic acid was added in various amounts to media containing 50 mM/100 ml. ethanol at pH 2.1; and similarly sodium acetate solution was added to media brought to pH 6.7.

The data in table III clearly show that at pH 2.1, 3 to 4 mM/100 ml. of

TABLE II

TOXICITY OF ETHANOL AT HIGH CONCENTRATION ON CELLS OF *H. Anomala.*

pH	Initial conc. ethanol mM/100 ml.	Final conc. ethanol mM/100 ml.	Ethyl acetate mM/100 ml.	Acetic acid mM/100 ml.	% dead cells[1]
2.1	80	56.5	1.50	2.86	20
	100	77.0	1.55	2.70	80
	120	94.7	.86	2.35	95
	143	125.6	.42	1.60	100
6.7	80	55.8	.03	4.90	5
	100	75.0	.04	5.70	0
	120	90.4	.06	5.20	0
	143	116	.44	3.56	5
	186[2]	165	.03	.99	5
	254[2]	228	.01	.28	95

[1]Determined by methylene blue 1:10,000 as vital strain. As previously noted (Tabachnick and Joslyn, 21), a plate count would probably show 20% more cells to be viable.

[2]Cells for these concentrations were obtained from a different crop grown in the same medium 1.

added acetic acid inhibited ethanol oxidation and ethyl acetate production. Vital staining showed 40% of the cells to be alive at 4 mM/100 ml. of acetic acid and only 5% at 5 mM/100 ml. In the control flasks without ethanol, 25% of the cells were alive and unstained at an acetic acid concentration of 3 mM/100 ml. and 5% at a concentration of 6 mM/100 ml. Judging from the observations with vital stain alone and acetic acid concentration of about 5 mM/100 ml. is toxic at pH 2.1. BEDFORD (**2**) also noted that acetic acid was toxic to most of the *Hansenula* species which he studied. PEEL (**18**) reported that at pH 4.6, 0.008 M acetate produced a 50% increase in ester production but that the amount of ester formed then decreased with increase in acetate added until at 0.1 M, ester formation was completely inhibited.

TABLE III

THE INHIBITORY EFFECT OF ACETIC ACID ON ETHYL ACETATE PRODUCTION AT pH 2.1 AND pH 6.7. (INITIAL ETHANOL CONCENTRATION = 50 mM/100 mL)

pH	Initial acetic acid mM/100 ml.	Final acetic acid mM/100 ml.	Ethyl acetate mM/100 ml.	Ethanol remaining mM/100 ml.
2.1	1.0	1.75	7.50	16.3
	2.0	2.46	5.30	20.0
	3.0	3.72	.33	41.5
	4.0	3.90	.03	45.0
	6.0	5.26	.02	45.0
	1.0[1]	.12	.00	....
	3.0[1]	2.60	.02	....
	6.0[1]	5.40	.02	....
6.7	9.0	9.8	.05	19.3
	13.0	11.1	1.45	15.2
	21.0	20.6	1.70	14.6
	34.0	33.0	1.81	12.1
	9.0[1]	.23	.00	....
	13.0[1]	.35	.00	....
	17.0[1]	1.30	.00	....
	34.0[2]	....	.00	....

[1]Control flask without added ethanol.
[2]Control flask without yeast cells.

Such a stimulatory effect may occur as our yield of 7.5 mM/100 of ester in the presence of 0.01 M acetic acid is unusually high for these experiments which averaged 5 to 6 mM/100 of ester from 50 mM/100 ethanol. GORDON (**8**) found 1% acetic acid to be toxic to germinating spores of an ethyl acetate producing ascomycete, *Endoconidiophora moniliformis* (Hedge) Davidson.

Table III also shows that 1 mM of added acetic acid can be utilized at pH 2.1, but that higher concentrations were toxic to the cells and remained unchanged. At a pH of 6.7, 15.7 mM/100 ml. of acetate were utilized by this organism. Contrary to the reports of some investigators (**9, 22**), acetic acid cannot be utilized as a sole carbon source for ethyl acetate production by *H. anomala*.

Acetate at concentrations as high as 34 mM/100 ml. had no effect on ethanol utilization by the cell suspensions at pH 6.7. Control flasks without ethanol showed a utilization of acetate (the pH in these flasks rising to 8.2 to 8.9). In the presence of comparable concentrations of both ethanol and acetate at a favorable pH, *H. anomala* utilizes the ethanol much more rapidly than acetate.

Preformed acetic acid apparently is not the source of the acid portion in the ethyl acetate molecule, with *Hansenula* (**24**) and also not with *Acetobacter xylinium* (**7**).

EFFECT OF VARIOUS INHIBITORS.—That ethyl acetate may be formed at pH 6.7 but is then rapidly hydrolyzed by *H. anomala* esterase, was shown by adding two specific esterase inhibitors,[2] di-isopropyl fluorophosphate (DFP) and tetra-ethyl pyrophosphate (TEPP). Other inhibitors (KCN, CH_2ICOOH, NaN_3, NaF, and $NaHSO_3$) were also tried, but the most significant results were obtained with the inhibitors listed in table IV.

TABLE IV

EFFECT OF VARIOUS INHIBITORS ON ETHYL ACETATE PRODUCTION AT pH 3 AND pH 6.7. (INITIAL ETHANOL CONCENTRATION = 25 mM/100 ml.)

pH	Inhibitor	mols/liter	Ethyl acetate mM/100 ml.	Ethanol remaining mM/100 ml.	Acetic acid mM/100 ml.
3	$NH_2OH \cdot HCl$	1×10^{-3}	2.75	0.6	.43
	DFP	1×10^{-3}	2.25	7.9	.46
	TEPP	1×10^{-3}	1.80	11.3	.43
	Control (no inhibitor)	....	2.05	0.3	.20
6.7	$NH_2OH \cdot HCl$	1×10^{-2}	.74	2.2	3.44
	DFP	5×10^{-4}	1.80	4.1	1.89
	TEPP	1×10^{-4}	.89	5.5	2.54
	Isopropanol control	2.6×10^{-2}	.68	7.5	2.70
	control (no inhibitor)	....	.84	5.0	1.62

Ethanol at a concentration of 25 mM/100 ml. was used in these experiments, and one experiment was conducted at pH 3 rather than 2.1 to minimize toxic effects of the products. DFP and TEPP were dissolved at a concentration of 0.1 M in isopropanol;[3] the other inhibitors were added as 0.1 M water solutions. The experiment was carried out for 24 hours.

No inhibition of ester synthesis or hydrolysis was observed at pH 3 with any of the inhibitors used. Hydroxylamine may be a possible exception, since a different type of odor was emitted from the flasks containing it. It is possible that an acetylated hydroxamate may have been formed, as there

[2] We wish to thank Dr. A. K. Balls for generously donating these compounds.

[3] A 1% water solution of DFP is completely hydrolyzed within 72 hours (10^{-3} M DFP = 0.18%). The half-life of TEPP in water is 7 hours at 26° C (KILBY, 13).

was 36% more ester in its presence than in the control. DFP resulted in a slight increase of ester above that in the control but the results were not significant, probably because of rapid hydrolysis of these inhibitors at pH 3.

At pH 6.7, a large increase in ethyl acetate was observed in the presence of 5×10^{-4} M DFP. It has already been shown (table III) that an excess of acetate at this unfavorable pH for ester production also resulted in an accumulation of ester. The accumulation of ester in the presence of DFP at pH 6.7, is additional evidence that ester formation cannot be the result of the reversal of enzyme catalyzed hydrolysis. PEEL (**17**) found increased accumulation of ester in the presence of DFP at lower pH.

ETHYL ACETATE UTILIZATION.—The utilization experiments were conducted in one-liter rubber-stoppered flasks. Toxic concentration of ethyl acetate for the yeast cells used appeared to lie between 0.14 M (at pH 3.1) and 0.20 M (at pH 6.7). WILL (**25**) found the strain of *Willia anomala* which he used could grow in 4% (0.46 M) ethyl acetate. That hydrolysis preceded ester utilization, was shown by temporary accumulation of free acetic acid, which appeared in the medium but was then rapidly utilized, but ethanol was not found even in early stages. The addition of 5×10^{-4} M DFP and TEPP resulted in a 28% inhibition of ester utilization. The optimum pH for ethyl acetate utilization was about pH 7. This experiment confirmed the observation that the accumulation of ethyl acetate at low pH was in part due to the inhibition of esterase activity of the cells.

UTILIZATION OF OTHER ALCOHOLS.—There are conflicting reports in the literature (**18, 24, 26**) as to the possibility of ester production from propanol, butanol or amyl alcohol. All of the straight chain alcohols up to C_5 were tested; isopropanol, isobutanol, isoamyl, and benzyl alcohols were included also. At pH 3, all of these alcohols with the exception of ethanol and isopropanol were toxic at a concentration of 2 gm./100 ml. In the spent isopropanol medium, the presence of acetone was detected as the 2,4-dinitrophenyl hydrazine derivative.

This experiment (without benzyl alcohol) was then repeated at alcohol concentrations of 0.5 gm./100 ml. and 0.1 gm./100 ml. at pH 3 and pH 6 with the shaking time reduced to 12 hours in an attempt to detect any ester formed before it was utilized. In a separate experiment at pH 3 with the 0.1 gm./100 ml. concentration of alcohols, the cell suspension density was also reduced to 5 mg. cell nitrogen/100 ml. Although the alcohols were completely utilized ($\pm$ 10%), no ester formation could be detected in any of these experiments, thus confirming the observations of PEEL (**18**). The alcohols, however, were oxidized by *H. anomala* to their corresponding acids and small amounts of these volatile acids accumulated in the medium. The production of these fatty acids from their corresponding alcohols probably contributed considerably to the toxicity of these alcohols, especially at pH 3. Acetic acid was also detected during the utilization of butyl and amyl alcohols.

CARBON BALANCE FOR ETHANOL UTILIZATION.—Carbon balance with cell

suspensions was made with 500-ml. Erlenmeyer flasks so constructed,[4] TABACHNICK (**20**), that at the conclusion of an experiment the gases formed could be displaced into an Orsat gas analysis apparatus by running mercury in through the bottom of the flasks. These flasks were closed with rubber stoppers which were wired in before placing the flask on the shaking machine. Into these flasks were placed 20 ml. of medium at pH 2.1 containing approximately 5 mM of ethanol and a cell density equivalent to 2.2 mg. of cell nitrogen in 20 ml.

A typical carbon balance is shown in table V. The carbon recovery was 65.6%. The unaccounted-for carbon is probably present in the cells as some assimilation product. An average of six such determinations gave a carbon recovery of 60%. The oxidation reduction (OR) values in column 4, table V, were calculated as follows: the number of oxygen (O) atoms in a compound minus one-half the number of hydrogen (H) atoms in the compound is equal to the oxidation reduction value. The algebraic sign of the OR value is changed when the particular compound is utilized as a source of hydrogen or oxygen (**12**). The redox index value (column 5) is obtained by multiplying the millimoles of the compound utilized or produced by its oxidation reduction value. The redox index is the ratio of the sum of products of oxidation (+) to the products of reduction (–). A perfect redox index equals one, since oxidation is accompanied by equivalent reduction (**6**). Our redox index is high but it is extremely doubtful that any major product has been overlooked. The high redox index may be due to the inability to account for the formation of some reduced product of assimilation present within the cells, although the accepted assimilation formula (CH_2O) with an OR value of zero, would make this suggestion unlikely. An attempt was made to inhibit the assimilation of ethanol by using concentrations from

TABLE V

CARBON BALANCE FOR ETHANOL UTILIZATION BY CELL SUSPENSIONS OF *H. Anomala*.

Compound	Millimols	Millimols carbon	OR value	Redox index value	mM/100mM ethanol utilized
Utilized					
Ethanol	3.28	6.56	(+) 2	+ 6.56	100
Oxygen	2.62		(–) 2	– 5.24	80
Products					
Carbon dioxide	1.08	1.08	(+) 2	+ 2.16	33
Acetic acid	.053	.106	0	0	1.6
Ethyl acetate	.78	3.12	(–) 2	– 1.56	23.8

Time = 24 hours. Cell density = 2.2 mg. cell N per 20 ml.
Temperature = 23–26 °C. Ethanol = 5 mM/20 ml.
pH = 2.1
C recovered = 65.6% redox index $= \frac{+8.72}{-6.80} = 1.28$.

[4] We wish to thank Mr. Floyd Stadtman for modifying these flasks.

5×10^{-5} M to 3×10^{-4} M 2,4-dinitrophenol, but either no inhibition occurred or complete inhibition of respiration resulted.

CELL-FREE EXTRACTS.—Various methods were tried for breaking the yeast cell wall to release the cell contents; grinding the frozen cells with powdered Pyrex glass, UMBREIT *et al.* (**23**), was found to be most promising. Using Warburg flasks, the milky cell-free extract was qualitatively shown to take up oxygen and to decolorize methylene blue with ethanol as substrate. Dilution of the extract to one-third resulted in a complete loss of oxidative activity. Although various combinations of substrates, co-factors and inhibitors were tried at various pH levels both aerobically and anaerobically, TABACHNICK (**20**), little or no ester could be detected in the cell-free extract experiments. The presence of a very active esterase in these extracts appeared to be the major difficulty in attempting to show ester synthesis. The addition of approximately 0.01 M DFP to the extracts failed to inhibit the esterase sufficiently. The use of hydroxylamine as trapping agent for the activated carbonyl portion of an acetylating mechanism (**10, 14**) gave promising results but this remains to be studied further.

Discussion

The use of ethanol as the sole carbon source for ethyl acetate production by cell suspensions of *H. anomala* eliminated the need for an anaerobic metabolic phase found necessary when glucose is used. The only compounds formed in significant amounts from ethanol are carbon dioxide, acetic acid and ethyl acetate.

Although there is but little evidence that acetaldehyde is an intermediate in ethyl acetate formation by *H. anomala*, there is some chemical evidence (**5**) that ethanol can be directly converted to ethyl acetate at 275° C with Cu and Ce as catalyst and activator respectively, via acetaldehyde. Acetaldehyde has been well established as an intermediate in ethanol oxidation to acetic acid by microorganisms (**16, 19**). Acetaldehyde has also been detected by GRAY (**9**) in an *H. anomala* medium containing ethyl acetate, and was shown by PEEL (**18**) to stimulate ester formation from ethanol.

The inhibition of the esterase at pH 2.1 and the production of ethyl acetate at pH 6.7 in the presence of DFP show clearly that the esterase is not involved in ester synthesis. Ester synthesis appears to follow a separate pathway from hydrolysis. Our data confirm PEEL (**18**) who concluded that the acetate portion of the ester must arise from acetate formed within the cell and not from free acetic acid present in the medium. The accumulation of acetate in the medium is usually a sign of ethyl acetate hydrolysis rather than ester synthesis. Experiments in the literature which show ester synthesis as a reversal of hydrolysis, such as the production of acetylcholine by acetylcholine esterase (**10**) or a diglyceride by *Ricinus* lipase (**1**), were conducted *in vitro* with high, unphysiological concentrations of alcohol and acid. With the proper esterase inhibitors it should be possible to show that the formation of these compounds *in vivo* does not involve esterase activity.

H. anomala can carry out an incomplete oxidation of ethanol which

ordinarily would result in the accumulation of toxic amounts of acetic acid at the acid reaction in which this organism is usually found. The amount of free acetic acid in the medium at low pH is usually below 0.01 M due to the ability of this organism to produce ethyl acetate. Without this mechanism for ester formation, *H. anomala* could not long survive below pH 4. Although this may be an inefficient means for ethanol utilization, eventually the ester and acetate are utilized and CO_2 and water are the final end-products.

At pH 6.7 little or no ester is formed, and *H. anomala* resembles physiologically members of the genus *Brettanomyces*. As has been found (**11**), if the acetate in the ester is included, *H. anomala* at pH 5.5 produces the highest acid yield of any of a large number of yeast species not excluding members of the genus *Brettanomyces*. Custers (**4**) has shown that *B. claussenii* produces a 33% yield of acetic acid from 2% glucose at pH 6.4 after 90 hours of aeration. Interestingly enough, the acetic acid was formed after the ethanol had accumulated. However, his experimental conditions were such that he could not be sure that all of the acetic acid arose from ethanol oxidation. Similar results would be expected at pH 6.7 with cell suspension of *H. anomala*, and if it were not for the characteristic ogive cells of *Brettanomyces* and the lack of spore production, species of this genus could easily be confused with *H. anomala*. *H. anomala*, however, grows rapidly and is long lived, all species of *Brettanomyces* grow slowly and are short lived. Many of the colonies giving the clear zones typical of *Brettanomyces* on $CaCO_3$, glucose agar have later been identified as *Hansenula* species (**15**). Species of *Brettanomyces* do not produce ester nor is it known whether members of this genus have an active esterase. It would be of interest to observe whether the addition of DFP to a suitable *Brettanomyces* culture would result in the formation of ester by this organism.

Summary

Using cellular suspensions of *Hansenula anomala* with ethanol as substrate, the optimum pH for ester accumulation was found to be between 2.1 and 2.6. The high accumulation of ethyl acetate at low pH is in part due to the inhibition of esterase activity. At pH 2.1, acetic acid was found to be toxic at concentrations ranging from 0.01 M to 0.05 M. At pH 6.7, on the other hand, cellular suspensions produced 70 mM of acetate per liter from ethanol without apparent toxic effect and could oxidize added acetate in concentrations as high as 0.3 M. Although only trace amounts of ester were formed from ethanol at pH 6.7, the addition of DFP, a specific esterase inhibitor, resulted in a significant accumulation of ester at this pH, suggesting that ethyl acetate may be formed and then rapidly hydrolyzed by the cells at pH 6.7.

Formation of esters from propanol, butanol, and amyl alcohol could not be detected.

From the high yields of ester formed, the experiment with DFP at pH

6.7 and the observation that ester formation is linked to respiration, it is evident that ethyl acetate formation by *H. anomala* is most probably an energy coupled reaction and is not the result of a reversal of a simple hydrolysis mediated by an esterase.

Attempts to obtain an active cell-free preparation which synthesizes ester were unsuccessful. A major difficulty was the presence of the esterase in these extracts.

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FURTHER EVIDENCE OF PERSISTENCE OF THE 2,4-D STIMULUS IN COTTON[1, 2]

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Received December 10, 1952

Although several reports in the literature indicate that 2,4-dichlorophenoxyacetic acid (2,4-D) or the stimulus therefrom does not normally persist in plants (**3, 4, 5, 6, 15**), it has been demonstrated by the authors that cotton is an exception to this generalization (**8, 11, 13**). This fact became apparent upon demonstration of a transmission of the stimulus into seed embryos formed subsequent to 2,4-D application. In studies (**7, 11, 12**) dealing with the effects of 2,4-D on the vegetative characters of the cotton plant, it appears that persistence is also shown. This paper deals with further experiments designed to show the continuing activity of 2,4-D in vegetative organs of the cotton plant as evidenced by (a) malformation and (b) recovery of the growth substance.

Experimentation and results

Plants in experiments 1 to 3 of this investigation were grown in 2- or 3-gallon jars of fertile Houston Black clay at College Station, Texas. Aqueous solutions of the sodium salt of 2,4-D, expressed in acid equivalent amounts, were utilized in all instances. Grasselli spreader was added at the rate of 2 ml. per liter of solution. The plants utilized in experiment 4 were grown in flats of fertile loam at Chicago, Illinois. The free acid of 2,4-D was utilized in this latter experiment. In all experiments the plants were grown under greenhouse conditions.

EXPERIMENT 1.—This experiment was performed to observe the malformation of leaves, both as to degree and number, on the main stem of cotton plants treated with known amounts of 2,4-D. Seeds of Stoneville 2B cotton, *Gossypium hirsutum* L., were planted February 27, 1950, and a series of five uniform plants was treated at the developmental stages of anthesis (May 5) and fruiting (May 20) with 0.04 mg. of 2,4-D per plant. This amount of 2,4-D was applied in 5 ml. of solution by spraying the entire plant, care being taken to prevent run-off. These two developmental stages were as characterized in previous publications (**12, 13**). At the time of treatment,

[1] This work supported in part by the Texas Agricultural Experiment Station and a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[2] Published with the approval of the Director of the Texas Agricultural Experiment Station as Technical Article no. 1650.

plants sprayed at anthesis had produced 13 main stem leaves while those sprayed at the fruiting stage had 17. By July 7, plants of both series had produced a mean of 23 main stem leaves and a majority of the bolls on the plants had matured. Following boll maturity the plants again characteristi-

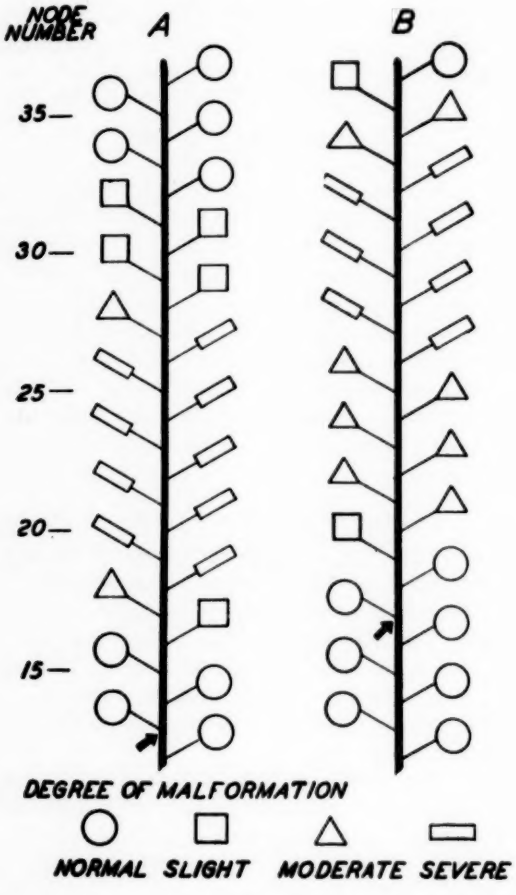

FIG. 1. Diagrammatic representation of malformation of leaves on main stem of Stoneville 2B cotton plants treated at anthesis (A) and fruiting (B) with 0.04 mg. of 2,4-D. Arrows indicate terminal macroscopic node at time of treatment.

cally exhibited rapid main stem elongation and leaf initiation. On September 22, plants of both series had produced a mean of 33 main stem leaves and were in their second flowering period. By December 1 only three additional main stem leaves per plant had been produced of which only the latest formed was free of malformation. During the time that this experiment was

in progress, plants treated at anthesis produced a mean of 16 malformed main stem leaves and those sprayed at fruiting had produced 17. The degree of malformation of these leaves is illustrated in figure 1. The various degrees of severity are as characterized in a previous publication (**12**).

This experiment was repeated in the period between November 27, 1950, and June 1, 1951. The plants grown during this time produced a mean of 15 malformed main stem leaves. The pattern of leaf malformation was similar to that observed in the earlier experiment (fig. 1).

EXPERIMENT 2.—Since it has been pointed out by numerous workers that the major portion of 2,4-D applied to leaves is taken up within a short time after application (**2, 14, 17**), it would appear that the response observed in experiment 1 was sufficient to demonstrate the persistence of the 2,4-D stimulus. The possibility existed, however, that the response was not due to persistence but to continued 2,4-D absorption from the initial leaf surface application. An additional factor which might account for the above noted response would be the presence of sufficient leaf primordia within the terminal bud to delay the appearance of an effect that occurred at the time of 2,4-D application. Although this latter possibility seemed remote on the basis of published data concerning vegetative buds (**9**), it too could not be eliminated without checking. Consequently this experiment was conducted to determine whether or not these two factors had an influence on the apparent persistence. Stoneville 2B cotton plants were treated at the floral primordia stage (**12, 13**) on December 19, 1950, with 0.01 mg. of 2,4-D per plant. Application in this case was made within lanolin rings on the third and fourth true leaves, 0.05 ml. of a 100 p.p.m. solution being placed on each leaf. After intervals of 1, 2, 3, 5, and 12 days the treated leaves were removed from a series of plants by cutting the petioles at their junction with the leaf blade. The leaves on an additional group were allowed to remain on the plants for the duration of the experiment (82 days). Nine plants were included in each series. Plants of all series had ceased to produce malformed main stem leaves by March 11, 1951, at which time final observations were made. By this time, also, the plants had passed through one reproductive cycle and many bolls had matured.

Under the conditions of this experiment, it is apparent from the data in table I that 2,4-D absorption by the plants continued over a period of about five days. It was also observed that the number of malformed leaves formed was greater with application of 0.04 mg. of 2,4-D per plant than when 0.01 mg. was applied. With the 0.01 mg. application the maximum number of main stem leaves malformed was nine.

At the time of the 2,4-D application, dissection of terminal main stem buds of a series of plants indicated the presence of five to six leaves or leaf primordia. The largest two or three of these (maximum length approximately 11 mm.) possessed macroscopic petioles. By observing treated plants, it was determined that one or two of the larger leaves still within the bud at the time of treatment did not show any malformation upon expan-

TABLE I

INFLUENCE OF TIME ALLOWED FOR 2,4-D ABSORPTION ON NUMBER OF MALFORMED MAIN STEM LEAVES PRODUCED BY STONEVILLE 2B COTTON PLANTS.

Days	Number of malformed leaves	Total number of nodes	Leaves produced after treatment
1	1.1	19.2	10.8
2	2.0	20.8	12.4
3	4.7	19.4	11.1
5	7.9	18.9	10.3
12	8.0	19.7	10.9
82	9.0	19.8	10.9

sion. Assuming that the plants which were treated with 2,4-D had terminal buds equivalent to those dissected, the conclusion is indicated that the largest young leaf to be malformed was approximately 4 to 5 mm. in length, and the maximum number of leaves or leaf primordia present within the bud at the time of treatment that could be malformed was four or five.

EXPERIMENT 3.—Since it had been previously shown that the 2,4-D stimulus is translocated into the developing seed embryos during fruit development, it appeared desirable to determine the extent to which this stimulus would continue to make its presence evident in main stem leaf malformation if flowering was prevented. Thus a short-day variety of *Gossypium hirsutum* L., Marie Galante, was selected and grown in a 24-hour day by extending natural day length with incandescent lights. The seeds were planted on May 25, 1951, and the resultant plants treated on June 27. Two treated series of 12 plants each, receiving approximately 0.02 and 0.04 mg. of 2,4-D per plant, were utilized. The leaf blade dip method of 2,4-D application as described by EGLE and DUNLAP (7) was used. After five days the treated leaves were cut off midway along the petioles. Vegetative laterals produced by the plants during the course of the experiment were pruned off as they developed.

Dissection of terminal main stem buds at the time of treatment revealed six to seven leaves or leaf primordia present, the largest being 5 mm. in length. This was small compared with the young leaves of the Stoneville variety at time of treatment (11 mm.). The largest leaves present in the terminal bud of the Marie Galante variety were often observed to be malformed upon expansion, particularly with the 0.04 mg. application of 2,4-D. The sequence up the main stem of increasing severity of leaf malformation was similar to that previously indicated for the Stoneville variety (12). One significant difference in this instance was noted, however. With Marie Galante cotton, terminal stem growth did not cease once the leaves became very severely malformed, but rather leaf initiation continued, with as many as 16 or 17 very severely malformed leaves being produced before the start of a regressive series in severity of leaf malformation.

This experiment was terminated on September 14 at which time none of the treated plants were yet producing normal main stem leaves. In the

interval between treatment with 2,4-D and termination of the experiment (80 days) plants receiving 0.02 mg. or 0.04 mg. of the growth-regulator produced a mean of 26 malformed leaves (fig. 2). Of this number, 24 were produced after the 2,4-D treated leaves were removed from the plants. Of the malformed leaves produced, 13 and 16 for 0.02 and 0.04 mg., respectively, were of the very severe type. These latter figures do not represent the maximum number of very severely malformed leaves that would have

FIG. 2. Top portion of Marie Galante cotton plants, (A) treated with 0.04 mg. of 2,4-D and (B) untreated. Arrow indicates smallest expanding leaf at time 2,4-D treated leaf was removed from plant.

been produced had the plants been allowed to continue growth, for 18% of the plants of the 0.02 mg. series and 42% of those of the 0.04 mg. group were still initiating leaves with this degree of malformation at the termination of the experiment.

EXPERIMENT 4.—At the termination of the preceding experiment, the top two macroscopic nodes plus the terminal buds of plants of the 0.04 mg. series were preserved in anhydrous ether. On December 18, 1951, this material was ground in a mortar with 50 ml. of peroxide-free ether acidified with 2 ml. of 1 *N* HCl. After grinding, this material was placed in a stoppered

flask and allowed to stand at room temperature until January 28, 1952, at which time the ether was filtered off and made to volume. A known amount of lanolin was dissolved in an aliquot of this ether extract and then the ether was evaporated. Weighed amounts of the preparation were applied to the cotyledons of a series of 16-day-old Stoneville 2B cotton seedlings. The first true leaves of these seedlings were still within the apical bud at the time of the lanolin applications. The second day following treatment, the cotyledons showed moderate epinasty and in a week the expanding true leaves showed malformation. The leaf and stem malformations exhibited by these seedlings after a month were characteristic of those induced by 2,4-D (fig. 3).

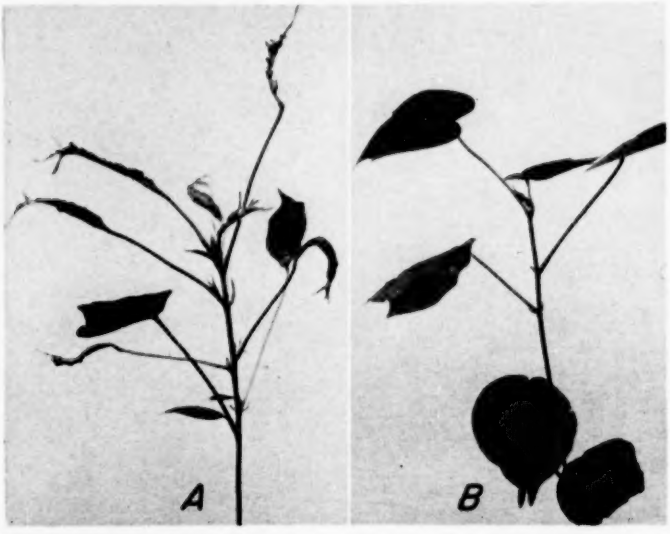

FIG. 3. Stoneville 2B cotton 30 days subsequent to treatment with (A) extract from 2,4-D treated Marie Galante plants and (B) extract from untreated Marie Galante plants.

Two and one-half months after the extract application, the top leaves of the treated plants were very severely malformed and the terminal meristems had ceased to initiate new leaves (**12**). In this interim the plants had produced a mean of seven leaves. After removal of the cotyledons to prevent contamination by any residual growth substance remaining in the lanolin smear, the shoots of these plants were extracted with acidified ether in the manner already described, an aliquot mixed with lanolin and smears were applied to a second series of seedlings. Again the seedlings exhibited characteristic 2,4-D malformation. One and one-half months later, the shoots of these seedlings (minus cotyledons) were harvested and extracted with acidified ether. These seedlings had also produced very severely malformed main stem leaves and terminal stem growth had ceased. A mean of seven

leaves per plant had been produced. Application of this extract to a third series of cotton seedlings likewise produced malformations characteristic of 2,4-D.

Extracts of normal plants were also made. When cotton seedlings were treated with these extracts, applied in lanolin, in no instance were any malformations observed.

Three months subsequent to the initial application of lanolin-extract mixture, another preparation was made from an aliquot of the Marie Galante extract to be checked against a series of lanolin applications containing known amounts of 2,4-D. Stoneville 2B cotton seedlings were again utilized in these bio-assays. After one month the plants treated with the extract were compared with those subjected to known amounts of 2,4-D. Using the degree of plant malformation as an index, it was calculated that the top two nodes plus the terminal bud of each of the Marie Galante plants (from experiment 3) yielded growth substance equivalent to 0.006 mg. of 2,4-D. If this growth substance was actually 2,4-D, it represents approximately 15% recovery of the amount originally applied to the leaves of the Marie Galante plants after they had grown for a period of three months and had produced 26 leaves. To eliminate the possibility that the observed effects were due to an endogenous growth regulator which had been greatly increased in concentration by 2,4-D application, determinations of the alkali and acid lability of the extract were made. After standing in 13 *N* sulfuric acid or after boiling in 1 *N* sodium hydroxide, no diminution of activity of the extracted substance was noted, indicating that this material was not any of the known naturally occurring growth substances (**17**).

Discussion

In this study it was found that the 2,4-D stimulus persisted in cotton plants for periods in excess of six months (experiment 1). As indicated by other workers (**5, 15**), this time interval is not too significant as an index to persistence if it represents a period in which the tissues being checked are in a relatively inactive or dormant state. In this instance, however, the cotton plants continued to exhibit growth. Demonstration of persistence of the stimulus in the vegetative growth of the Stoneville 2B variety was complicated by the reproductive phase, for fruiting results in reduced vegetative growth. With this curtailment of activity in the vegetative meristems and concurrent shift in loci of high metabolism to the reproductive structures, the 2,4-D stimulus is known to move into the developing bolls (**8, 11, 13**). In such cases, although the time interval over which the action of the growth regulator will show up is extended, the number of main stem leaves that will be malformed is not as great as if flowering had not occurred. This is illustrated in the results of experiment 3 in which a far greater number of main stem leaves were malformed in much less time.

It is quite obvious from this study that 2,4-D or its stimulus is long continuing in the cotton plant in that the number of malformed leaves initiated

greatly exceeded the number of leaves or leaf primordia present in the bud at the time of treatment with 2,4-D (experiments 2 and 3). With cotton, as was observed by WATSON (**16**) for beans, the larger leaves or leaf primordia present in the bud at time of treatment showed the least malformation. Because of the persistence of the 2,4-D stimulus in cotton, it is uncertain which size of leaves or leaf primordia are most vulnerable to the action of 2,4-D. It would seem, though, based on the sequence of leaf malformation, that the smaller the primordia the more severely it is affected. This malformation sequence, however, may be due to a greater amount of 2,4-D being present within the bud at the later periods of leaf initiation, thus the smallest primordia need not necessarily be the most vulnerable to 2,4-D action to obtain the results observed. The observation by BURTON (**1**) that more malformed leaves were initiated in cotton than observed in beans is undoubtedly accounted for, in part, by the longevity of the 2,4-D stimulus.

Whether a young leaf within the apical bud will be malformed by 2,4-D is determined to some extent by the amount applied (**12**). In experiment 3, for example, it was observed that 50% of the plants treated with 0.02 mg. of 2,4-D produced normal leaves after treatment, whereas only 8% of the plants subjected to 0.04 mg. did so.

The degree of persistence within the cotton plant (as measured by malformation of leaves) is to some extent determined by the amount of 2,4-D originally applied. For example, with Stoneville 2B cotton 16 to 17 main stem leaves were malformed if 0.04 mg. was applied whereas only 9 were distorted if the amount applied was 0.01 mg. (experiments 1 and 2). This is also shown in experiment 3 in which a greater percentage of the plants treated with 0.04 mg. of 2,4-D were showing very severely malformed terminal leaves at the close of the experiment.

In addition to the malformation of main stem leaves, persistence was demonstrated in this study by the extraction of a growth regulator from the meristems of treated plants. Even after long periods of growth, measured both in period of time and number of leaves initiated, a growth substance could be extracted which caused characteristic 2,4-D malformation when applied to other cotton plants. It was not definitely established that this regulator was 2,4-D, although the possibility of its being a naturally occurring auxin has been eliminated. The present observations, however, are very suggestive that the growth regulator is 2,4-D or some derivative thereof. On the other hand, since other compounds have been observed to induce 2,4-D-like leaf malformations in cotton (**10**), the possibility of its being some metabolite whose formation was 2,4-D-induced cannot be eliminated without further experimentation.

Summary

The persistence of the 2,4-D stimulus was demonstrated in the vegetative organs of two varieties of cotton, Stoneville 2B and Marie Galante. Evidence for this persistence was shown by (a) the period of time during which

the plants continued to produce malformed main stem leaves—up to six months, and (b) the number of malformed leaves produced—as many as 24. It was found that the number of malformed leaves formed was in excess of the number of leaves or leaf primordia in the terminal bud at the time of treatment, thus eliminating the possibility of an immediate effect not becoming evident for an extended time. That the persistence was not due to continued absorption of 2,4-D by the leaves was also established.

Persistence of the stimulus was further demonstrated by extraction of a growth-regulator from treated plants which induced cotton leaf malformations characteristic of 2,4-D. This regulator was extracted 80 days subsequent to the initial application of 2,4-D. Whether the growth-regulator was 2,4-D or a derivative therefrom was not clearly demonstrated. That this substance was not a naturally occurring auxin was established by its stability in alkali and acid solutions.

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PHOTOSYNTHESIS IN THE POTATO UNDER FIELD CONDITIONS[1]

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Received December 30, 1952

In spite of the importance of the potato as a food, little is known about the effect of environmental factors on photosynthesis in the plant. Studies with several other crops (**1, 12, 16, 23, 24**) have not only added to the knowledge of photosynthesis but have aided in understanding certain responses of these crops to environmental variations. VERDUIN and LOOMIS (**24**) and GABRIELSON (**10**) have shown that saturation light intensity for single, fully exposed leaves is reached at about one-fourth of full sunlight (10,000+ f.c.). THOMAS and HILL (**23**), working under field conditions, found directly proportional increases in CO_2 absorption with up to ten times normal (0.03%) CO_2 in the air. HOOVER, JOHNSTON, and BRACKETT (**14**) found increases in photosynthesis at light intensities less than 2,000 f.c. as the CO_2 content of the air was increased, with indications that both light and CO_2 supply may limit photosynthesis at low light intensities.

The temperature curve for CO_2 absorption under laboratory conditions indicates that the enzymatic reactions ($Q_{10} = 2$ or more) may limit the overall rate of the process (**9, 17**). In the field, however, Q_{10} values near 1.0 have been demonstrated for photosynthesis within the range of 2° to 40° C (**1, 6, 8, 23, 24**). Reduction in leaf hydration is assumed to check photosynthesis. SCHNEIDER and CHILDERS (**21**) found that CO_2 absorption of apple trees held in soil at the wilting percentage dropped to 13% of optimum. Recovery of optimum rates of CO_2 absorption occurred after a period of several days. VERDUIN and LOOMIS, (**24**) observed reduction to 5% of optimum in temporarily wilted maize leaves. Recovery occurred within a few hours after rewatering. MITCHELL (**18**) found that changes in humidity of surrounding air had little or no effect on photosynthesis. Stomatal closure without wilting did not markedly decrease CO_2 absorption. DASTUR (**7**) observed reductions in photosynthesis with a drop in water content of leaves.

The study reported here was planned to measure the effects of physiological and environmental factors on photosynthesis in the potato (*Solanum tuberosum* L.) under dry-land and irrigation conditions in western Nebraska. Data from earlier experiments have been reported by CHAPMAN (**6**).

Methods

Rates of CO_2 absorption were measured by determining the difference in CO_2 content between a measured stream of air and an equal volume of simi-

[1] Published with the approval of the Director as paper no. 595, Journal Series, Nebraska Agricultural Experiment Station.

lar air that had been drawn over an enclosed potato leaf. The apparatus was a modification of that described by HEINICKE and HOFFMAN (**12, 13**). Six units were operated simultaneously; two units measuring the CO_2 content of normal air streams, and four of air drawn over enclosed leaves.

A unit of the apparatus is diagrammed in figure 1. The leaf chamber (A, B, or C) enclosed a leaf in contact with the air stream. The CO_2 remaining in the air was absorbed in a column, H, of KOH solution containing 0.2 per cent. *n*-butanol. A coarse grade, fritted glass filter, G, served to break the air stream into small bubbles which gave CO_2 absorptions of 99 + per cent.

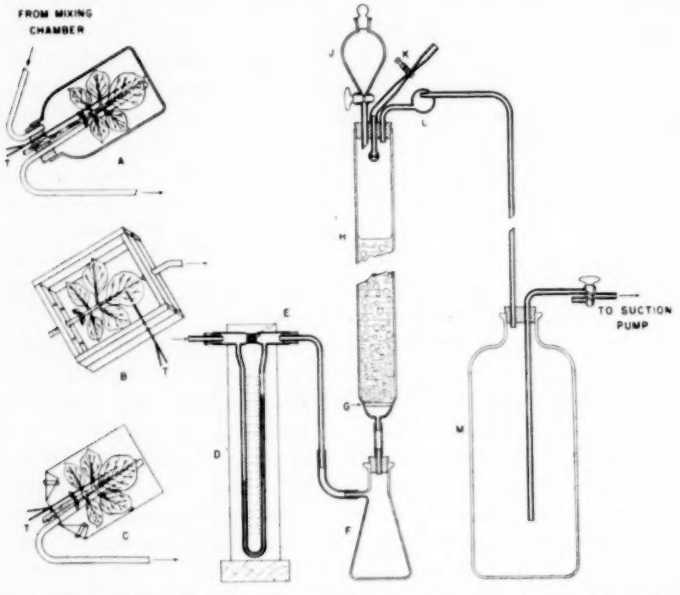

FIG. 1. Apparatus for measurement of CO_2 absorption. A, B, and C, leaf chambers used in different experiments; D, flowmeter; G, fritted glass disc to insure absorption of CO_2 in tower H.

Cellophane envelopes, C, were used as leaf chambers for the 1950 tests and for the soil moisture and wilting studies in 1951. Thin wooden frames, B, covered with cellophane, were used as leaf chambers for the 1949 tests. Two-liter clear glass, wide-mouth jars, A, were used as leaf chambers for the studies of varying CO_2 concentration. The system was made air-tight by sealing the leaf petiole into a split stopper with modeling clay.

During a test period, usually one hour, the flowmeters, D, were watched carefully to insure uniform movement of air through each tower. At the end of the period the absorbent solution was washed into the basal flask, F, an excess of $BaCl_2$ solution added, and the residual alkali titrated with stand-

ard HCl. Following the final test period for the day, the leaf chambers were removed, the leaves detached from the plants, and their areas determined with a planimeter from contact prints.

The apparatus diagrammed in figure 2 was used in conjunction with leaf chamber A (fig. 1) when higher than normal CO_2 in the air was to be used. Compressed CO_2, A, was reduced to low pressure, bubbled through concentrated H_2SO_4 in flask C and through water in flask D. Flowmeters, F, fitted with thermometer tubing were calibrated to pass sufficient CO_2 to raise the content of the air stream to the desired level. From the flowmeter the CO_2 was fed into the air stream inlet G, through the mixing chamber H, and then to the leaf chambers or control towers. The level of CO_2 supplied to any

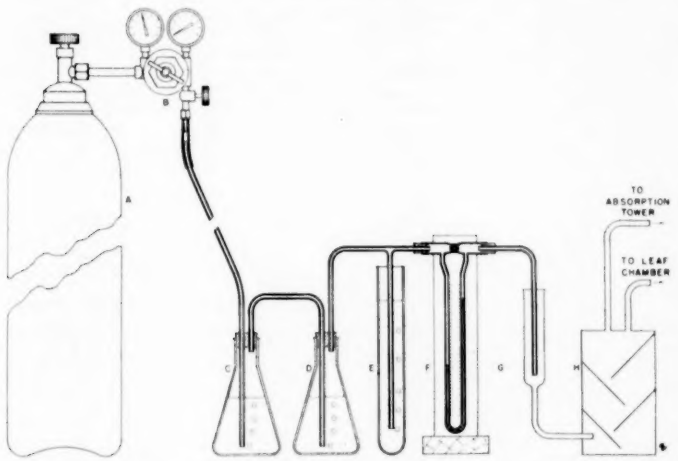

FIG. 2. Apparatus for enriching air with CO_2. Washed CO_2 from the cylinder, A, is metered into the air inlet, G, where 200 l. of air an hour is taken up for one control and one leaf-chamber air stream.

particular leaf was changed after each test period. Approximately ten minutes was allowed between tests for equilibration of the CO_2 level in the leaf chambers.

Light intensities were measured with a Weston photometer, the target of which was placed inside an empty leaf chamber held near the leaves and in the same plane in relation to the sun. Temperatures were determined with copper-constantan thermocouples (T, fig. 1) placed in the shade inside the leaf chambers.

All determinations were made near Alliance, Nebraska (elevation slightly over 1220 meters). The potatoes were planted about June 15th each year on summer fallowed land. A small number of plants were irrigated and fertilized with NH_4NO_3 in 1951 for the irrigation vs. dry-land comparisons.

Experimental results

Variability in rates of photosynthesis

Under controlled laboratory conditions other workers have observed fairly uniform rates of CO_2 absorption and have demonstrated rather characteristic responses of photosynthesis to variations in temperature, CO_2 supply, and light intensity. However, studies under more nearly natural conditions have frequently yielded widely variable responses not clearly attributable to changing external factors (**6, 12, 15**). Verduin and Loomis

TABLE I

VARIATIONS IN CO_2 ABSORPTION BY PAIRED LEAVES UNDER SIMILAR CONDITIONS IN 1949. DATA ARE MILLIGRAMS OF CO_2 ABSORBED PER SQUARE DECIMETER OF LEAF AREA PER HOUR.

Third leaf[2]			Fourth leaf[2]		
Plant A	Plant B	Per cent. variation[1]	Plant A	Plant B	Per cent. variation[1]
15.2	17.2	13	15.5	12.9	20
13.1	19.7	50	11.6	11.6	0
14.8	22.8	54	17.9	17.9	0
16.8	26.0	55	21.5	23.4	9
19.9	26.1	31	17.7	25.9	46
15.9	17.8	12	8.2	16.6	102
19.7	16.1	22	15.4	16.2	5
16.1	19.0	18	15.5	14.6	6
18.9	25.6	35	17.4	20.0	15
7.5	13.6	81	12.5	10.8	16
22.6	23.2	3	26.9	17.3	55
22.6	23.7	5	26.7	18.7	43
10.8	16.8	6	17.1	12.2	40
18.4	17.7	4	15.6	16.7	7
15.4	12.5	23	10.4	20.6	98
16.7	15.9	5	9.8	14.8	51
14.1	18.0	4	13.8	18.2	32
Mean					
16.4	19.5		16.1	17.0	

[1]Calculated by dividing the difference between A and B by the smaller of the two numbers and multiplying by 100.
[2]Coefficient of variability 95%.

(**24**) found variations of as much as 90%, averaging 25%, with paired maize leaves on the same or adjacent plants.

In these studies, the third and fourth leaves below the terminal leaf cluster on two similar plants were selected for determinations, and conditions were maintained as nearly identical as possible in all four leaf chambers. The results from several such paired observations are shown in table I. A maximum variation of 102% was recorded, variations of 40% or more were frequent, and a mean variation of 28% was found. Differences were as large between leaves on the same plant as between leaves on different plants, and

the coefficient of variability was 95%. The need for large numbers of observations from which to draw conclusions about even gross differences in photosynthesis under field conditions is clearly evident (cf. fig. 4).

The CO_2 content of normal air

The generally accepted average CO_2 content of the air is 0.03% by volume, or 0.594 mg. of CO_2 per liter of air. Analyses made at many different times and places have shown that this is not a fixed value but varies with a variety of factors. Since the quantity of CO_2 diffusing into the leaf cells may limit the rate of photosynthesis under field conditions, the importance of these variations in CO_2 content of the air is apparent. Böhning (**3**), for example, found that the rate of CO_2 absorption of apple leaves varied directly with the normal changes in CO_2 content of the atmosphere.

Heinicke and Hoffman (**12**) observed marked fluctuations in normal CO_2 from day to day, and state that the level was usually less in the afternoon than in the morning. They report an average value of 0.50 mg. of CO_2/l. of air. Verduin and Loomis (**24**) made hourly control-tower determinations of the CO_2 content of the air. They concluded that the usually accepted 0.03% is seldom reached during daylight hours at a height of 1 meter in an Iowa cornfield. Day to day as well as pronounced hourly fluctuations were also observed. They reported a maximum value of 0.80 mg./l. and a low value of 0.36 mg. of CO_2/l. of air. Graphs of their data showed a distinct downward trend during the early morning and a leveling of CO_2 content during the remainder of the daylight hours. Their means of 53 tests in 1939 and of 203 tests in 1940 were 0.488 mg. and 0.482 mg. CO_2/l. of air respectively. Typical, midday readings were 25% below readings made at dawn.

Although not designed to measure CO_2 content of the air specifically, the control-tower, sampling air usually 4 to 12 inches above the tops of the plants, measured its CO_2 content for the test period. In these tests the average CO_2 content of the air (mean of 222 tests) was 0.497 mg./l. in 1949, 0.484 mg./l. (148 tests) in 1950, and 0.482 mg./l. (177 tests) in 1951. These values indicate that the expected 0.03% by volume or 0.594 mg./l. was seldom obtained during daylight hours in a western Nebraska potato field.

A downward trend in CO_2 content of the air during the early daylight hours was clearly evident in most of our experiments. There was usually a gradual increase during the afternoon, when, as will be shown later, photosynthesis tended to drop with incipient or actual wilting. After sundown the CO_2 content generally rose rapidly to levels somewhat below 0.03%.

CO_2 concentration and CO_2 absorption

Increasing the CO_2 content of the air has been shown to increase photosynthesis when other factors are not seriously limiting (**4, 5, 14, 23**). For experiments with CO_2 concentration, a set of three leaves was selected on each of a number of days, and allowed to carry on photosynthesis with nor-

mal, twice normal, and five times normal CO_2 concentrations. The CO_2 level of the air moving to each leaf was changed after each one-hour test period; thus "fatigue," accumulation of photosynthate, and other effects were avoided or equalized among the test leaves. Each increment of 1.0 mg. CO_2/l. gave an increase of 14.2 mg. of CO_2 absorbed per dm.^{2} per hr. on July 31, a typical day (fig. 3). This regression coefficient is significant at the 1% level. Excellent correlation of these factors is indicated by the large coefficient of 0.89.

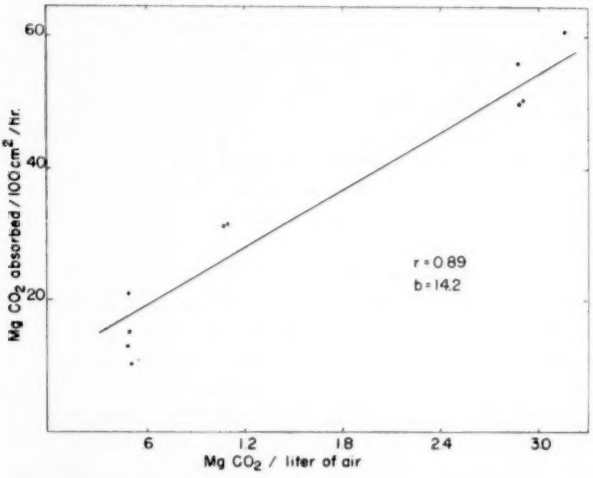

FIG. 3. Regression of CO_2 absorption by potato leaves on the CO_2 concentration in the air.

As shown in table II, the over-all average rates of CO_2 absorption increased directly with increased CO_2 content of the air. With normal CO_2, the higher average value in 1949, 18.2 mg./dm.$^2 \times$ hr. against 14.9 mg. in 1950 and 13.6 mg. in 1951, can be attributed to a large number of readings made on leaves of younger plants with their higher assimilation rates.

LIGHT INTENSITY AND PHOTOSYNTHESIS

Light intensities varied with the time of day and with cloudiness. Additional data at intermediate intensities were obtained by use of shades. Scatter diagrams of photosynthesis against light intensity suggested a break in correlation near 3000 f.c. with normal CO_2; near 4500 f.c. with twice normal CO_2, and near 6000 f.c. with five times normal CO_2. The data were accordingly arranged into groups ranging above and below these light intensity values, and regression and correlation coefficients were determined for the separate groups.

With normal CO_2, close correlation was found between light intensities below 3000 f.c. and CO_2 absorption, with highly significant r values of 0.80,

TABLE II

AVERAGE RATES OF CO_2 ABSORPTION WHEN LIGHT INTENSITIES WERE ABOVE SATURATION.

Year	CO_2 level	Mg./dm² × hr.
1949	Normal (*ca.* 0.25%)	18.2 ± .66
1950	Normal "	14.9 ± .87
1951	Normal "	13.6 ± .98
"	2 × normal (0.06%)	32.2 ± 2.63
"	5 × normal (0.15)%	50.0 ± 2.59

0.52, and 0.71 for data of three seasons representing 684 individual measurements. For light values over 3000 f.c. the corresponding r values were 0.04, − 0.04, and 0.09. The regression coefficients, b = 0.527, 0.347, and 0.351, show the gains of CO_2 absorption in mg. per dm.² per hr. for each added 100 f.c. of light up to 3000 f.c. In the groups with more than 3000 f.c. of light, the corresponding gains for the three seasons were 0.011, − 0.039, and 0.09, with no statistical significance. A scatter diagram of 1951 data with regression lines is shown in figure 4.

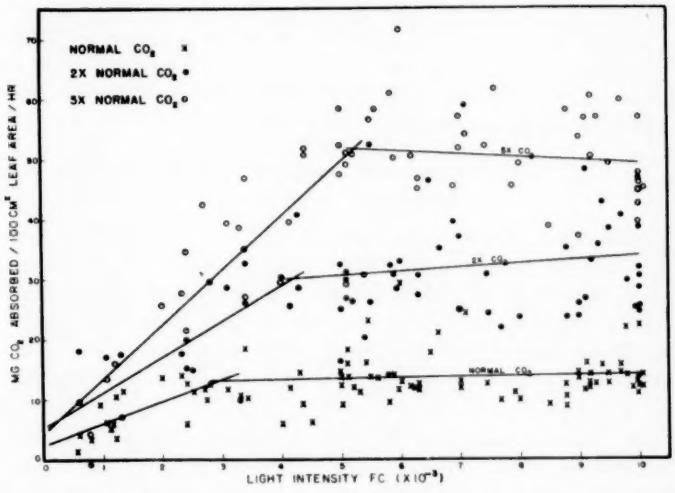

FIG. 4. Scatter diagrams and regressions for photosynthesis at varying light intensities and three levels of CO_2 in 1951.

With twice normal CO_2, the correlation values for the above and below 4500 f.c. groups were 0.139 and 0.745 respectively. The regression coefficients were 0.064 and 0.581 mg./dm.² × hr. × 100 f.c., only the latter, from the below 4500 f.c. group, was statistically significant.

The correlation values for the above and below 6000 f.c. groups (5 × normal CO_2) were 0.097 and 0.886. The regression coefficients were − 0.050 and 0.901 mg./dm.² × hr. × 100 f.c.; again only the values from the below 6000 f.c. group of data were statistically significant.

Light saturation of photosynthesis, as indicated by the intersection of these regression lines (fig. 4), was reached at about 3000 f.c. with normal CO_2; at about 4200 f.c. with twice normal, and near 5200 f.c. with five times normal CO_2. These intercepts are considered to be the best available estimates of light saturation for our experiments. In the 1949 data, for example, with 315 determinations, the intercept for normal CO_2 was at 2950 f.c., the same as for 1951. It is significant that even at light intensities below saturation, photosynthesis was increased by increasing the CO_2 content of the air, as indicated by the steadily increasing slope of the regression lines in figure 4.

The artificial division of our data must be emphasized. If all data points were plotted together they would give a curvilinear regression, as pointed out by SPOEHR (**22**), with less resemblance to a so-called limiting factor curve (**2**). Likewise, abscissa intercepts would approach values near zero in such a curvilinear plot.

The wide variations observed at similar light intensities indicate an interaction of factors influencing CO_2 absorption. At low light intensities, rate of CO_2 absorption was plainly retarded both by insufficient light and low CO_2 supply, with other factors causing the remainder of the observed variation. At light intensities above saturation the process was limited primarily by the CO_2 supply, and was influenced directly or indirectly by other factors to cause the observed wide variation. Among these other factors, protoplasmic hydration may be important.

THE TEMPERATURE COEFFICIENT OF PHOTOSYNTHESIS UNDER FIELD CONDITIONS

Photosynthesis is commonly reported to show a temperature coefficient of 2.0 or more, characteristic of enzymatically controlled reactions, under controlled laboratory conditions (**9, 17**). Temperature effects under more nearly field conditions tend to be obscured by other factors and complicated by the rapidly changing temperature of insolated leaves.

DECKER (**8**) found that temperature had no effect on photosynthesis in some conifers. VERDUIN and LOOMIS (**24**), working with maize leaves in the field, observed no correlation between moderately high temperatures and photosynthesis. THOMAS and HILL (**23**) also observed that the Q_{10} of photosynthesis was near 1.0 for several field crops over the range 16° to 28° C. ANDERSON (**1**) found a Q_{10} of 1.2 for photosynthesis in cereals over the range 2° to 12° C.

Our data have been divided into four groups on the basis of temperature for the three seasons (table III); all temperatures were measured with thermocouples inside the leaf chambers. Differences among the means for any one year or CO_2 level are not statistically significant. Although the highest rates of CO_2 absorption with normal air were recorded in the 27° to 32° C range, the data indicate that temperature did not significantly influence the trend of CO_2 absorption.

With the exception of Anderson's work, observations on the effect of temperature under field conditions are lacking at lower temperatures. We can say, however, that the range of temperatures usually encountered during the growing season does not appear to have a significant effect on photosynthesis, even with CO_2 increased to 5 × normal. This response is considered to indicate that CO_2 limits photosynthesis in the field.

Hydration and CO_2 absorption

Changes in hydration of cells and leaves are influenced both by the surrounding atmospheric conditions and by the availability of soil moisture. In general, the effects of hydration on photosynthesis have received less attention than the factors of temperature, CO_2 supply, and light, yet under field conditions the water factor must be considered of primary importance.

The mean rate of CO_2 absorption by wilted potato leaves in 1949 was 8.9 mg./dm.^{2} × hr., while the mean for turgid leaves under otherwise similar

TABLE III

MEAN RATES OF CO_2 ABSORPTION IN MG. PER DM^2 PER HR. WITHIN VARIOUS TEMPERATURE RANGES.

Year	18 to 27°	27 to 32°	32 to 38°	over 38°
1949	14.7	16.1	15.3	
1950	14.2	15.1	14.8	
1951		16.1	12.7	13.2
		2 × normal CO_2		
1951		30.3	29.6	33.3
		5 × normal CO_2		
1951		53.5	48.0	58.6

conditions was 16.5 mg. In 1951, irrigated and dry-land plants (16.3 inches average rainfall) were grown in adjacent plots. The mean rate of CO_2 absorption for the leaves from dry-land plants was 9.2 mg./dm.^{2} × hr., while the mean rate for the irrigated leaves was 14.0 mg. Test plants were within 25 feet or less of each other and leaves of similar age and size were selected. Representative data showed a more rapid diurnal decline in the absorption by dry-land leaves, following nearly equal early morning values. On cloudy or cool days this decline began several hours later, or on some days did not occur at all.

Also, in 1951, single plants were grown in cans 9 by 15 by 30 inches deep, which were buried in the ground to within about 2 inches of the top. The soil in a number of these cans was allowed to dry to the wilting percentage and rewatered following various periods of permanent wilting of the potato plants. Leaf turgidity was always regained within one hour after watering, while CO_2 absorption returned to normal more slowly. Following approximately 24 hours of permanent wilting, CO_2 absorption by leaves was 75%

of controls within one and one-half hours after rewatering (fig. 5). Complete recovery had occurred within four hours. The rate of CO_2 absorption by leaves which had been permanently wilted for approximately 72 hours had not returned to normal 6 hours after rewatering, when tests were terminated for the day. Some 48 hours later CO_2 absorption by different leaves from these same plants was equal to the unwilted controls.

Our results are in agreement with those of VERDUIN and LOOMIS (**24**) and also with those reported by SCHNEIDER and CHILDERS (**21**). Rate of recovery of photosynthesis by potato leaves following watering was dependent upon the duration of the period of moisture stress.

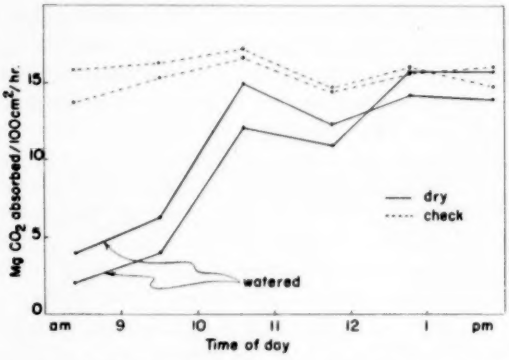

FIG. 5. Recovery of CO_2 absorption by potato leaves following 24 hours of permanent wilting.

VARIETAL DIFFERENCES IN CO_2 ABSORPTION

Data compiled by RABINOWITCH (**20**) indicate wide variation among plant species in rates of CO_2 absorption. However, most workers have been unsuccessful in measuring differences between varieties within a species with the techniques now available (**6, 19**).

In 1949 and 1951, the mean rate of CO_2 absorption by Triumph was significantly lower than for Cobbler, Progress, White Cloud, or Neb. 85.41-1. In 1950, the means were not significantly different. Although the mean for Triumph was lower in all three years, these values may be associated with its tendency to wilt more readily and for longer periods during midday than the other varieties tested. Since no significant differences appeared among the other varieties, it seems likely that nearly equal rates of CO_2 absorption per unit area of leaf might be expected under conditions of optimum hydration. Perhaps this optimum hydration is higher for Triumph, or perhaps moisture is lost more rapidly from Triumph leaves than from leaves of the other varieties tested.

Discussion

The CO_2 absorption method of measuring net photosynthesis has the advantage of eliminating translocation errors and permits use of the same

leaf in successive experiments. The principal difficulties involved are the accurate metering of the airflow through the absorption units and prevention of excessive temperature buildup in the leaf chambers.

Variations in rate of CO_2 absorption observed under apparently identical external conditions may be attributed to poorly understood internal factors which differ for different leaves. Probably important among these factors is hydration of the protoplasm. Although we observed large variations in net CO_2 absorption, we did not observe the release of CO_2 by leaves in light reported by some of the earlier workers (**15**). The variability of their data on CO_2 of the air leads one to suspect that some experimental variable was responsible for these reported instances of negative photosynthesis.

An interesting by-product of the air stream method was the data on CO_2 content of the air. Values of 0.594 mg./l., the usually accepted average CO_2 content of the air, were practically never reached under field conditions in western Nebraska. Early morning and late evening values were considerably higher than the midday CO_2 content. The consistently low values obtained in daytime analyses suggest that the lower layers of air above the plants become noticeably depleted of CO_2 during photosynthesis. Two factors tend to reduce this depletion: mixing of depleted air with air of higher CO_2 content and CO_2 evolution by respiring plants and soil organisms. These western dry-land soils are lower in organic matter and generally drier, and night temperatures are much lower than at Ames, Iowa, which may explain why early morning values were not as high as those reported by VERDUIN and LOOMIS (**24**).

The fact that photosynthesis in the potato was doubled by doubling the CO_2 content of the air, and increased nearly four times by increasing the CO_2 to 0.15% in short time experiments, indicates the importance of the finding that average daytime CO_2 content of the air is 20 to 25% below the accepted normal. Although THOMAS and HILL (**23**) failed to obtain sustained increases of photosynthesis with added CO_2 in their 6×6 ft. greenhouses, the possibility remains that dry matter production by plants under otherwise optimum conditions is limited by the availability of CO_2.

Limitation by CO_2 supply is assumed to explain the low temperature coefficient of photosynthesis in the field, averaging near 1.0, in contrast to coefficients of 2 and more that have been reported in the laboratory with CO_2 concentrations 100 to 200 times normal.

Light saturation of photosynthesis was reached at about 3000 f.c. with normal CO_2 supply, at about 4200 f.c. with twice normal CO_2, and at near 5200 f.c. with five times normal CO_2. At light intensities below saturation, both low CO_2 supply and insufficient light limited the rate of the process. These values suggest that midday light intensity is seldom a seriously limiting factor for photosynthesis in the field. While the lower leaves of trees or thickly planted crops may receive less than optimum light (**11, 23**), the light intensity under many planted crops varies from 2000 to 4000 f.c.

Wilting was nearly always attended by marked reductions in rate of CO_2 absorption. This reduction may be a result of mechanical interference with

CO_2 entrance or it may be a result of physiological changes interfering with the photosynthetic reactions. After having been nearly equal during the early daylight hours, the midday rate of CO_2 absorption by leaves of dry-land plants was well below that for leaves of irrigated plants. This response seemed to be correlated with availability of soil moisture and severity of atmospheric conditions. Permanent wilting of leaves apparently caused structural or protoplasmic changes from which the leaves recovered fully only over a period of hours or days.

In spite of the fact that varieties differ greatly in total yield of dry matter, vine size, date of maturity, and leaf type, we have not been able to measure differences in their rate of CO_2 absorption per unit leaf area. The magnitude of such differences, if they actually exist, must be small.

Summary

The average variability in rates of CO_2 absorption by paired leaves of potato under field conditions was 28% and the coefficient of variability was 95%. In spite of such variability, evolution of CO_2 from leaves in light was not observed, even on permanently wilted plants.

The CO_2 content of field air averaged about 0.025% during the day with higher values at night, but still generally below 0.03%. Over short periods, photosynthesis was increased directly by increasing CO_2 to 0.15%.

With normal CO_2, photosynthesis showed light saturation in fully exposed leaves at a light intensity of 3000 f.c.; with 2× normal (0.06%) CO_2 an intensity of 4200 f.c. was required for saturation, and with 5× normal (0.15%) CO_2, 5200 f.c.

Rates of photosynthesis with normal air averaging 0.025% CO_2, were about 14 mg./dm.^{2} × hr., or 1.4 gm./m.^{2} × hr., equivalent to 0.96 gm. glucose/m.^{2} × hr. With CO_2 at 0.06% the average rate was 32 mg./dm.^{2} × hr., and at 0.15% it was 50 mg.

Even with 5× normal CO_2, the maximum concentration used, photosynthesis showed a temperature coefficient of approximately unity over the range 18 to 40° C. This lack of temperature response is assumed to be due to the limiting levels of CO_2 present in the field.

Lower mean rates of CO_2 absorption by Triumph leaves were associated with its tendency to wilt more readily than the other varieties tested. We have been unable to measure inherent varietal differenes in CO_2 absorption by the air stream method, if such differences actually exist.

Photosynthesis in wilted leaves averaged half that in nonwilted leaves. After watering permanently wilted plants, the rate of recovery of photosynthesis by leaves was directly dependent upon the duration of the period of wilting.

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ABSORPTION AND METABOLISM OF TRITIUM OXIDE AND TRITIUM GAS BY BEAN PLANTS[1]

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Received February 16, 1953

Introduction

Tritium (H^3) is a radioactive isotope of hydrogen, having a half life of 12.4 years and emitting 0.019 mev. beta particles. Due to its radioactivity, accurate measurement of this tracer can be made even when small quantities are present.

The experiments described in this paper were designed to evaluate the extent of incorporation and retention of tritium in bean plants which had been grown in a nutrient solution containing tritium oxide. The tritium oxide content of the plant water and the tritium organically bound in the various plant tissues were determined under several environmental conditions. A brief study was also made of the incorporation of tritium by a bean plant when exposed to an atmosphere containing tritium gas. The molecules containing tritium were HTO and H-T, respectively.

Little work has been published on the absorption and metabolism of tritium oxide in plants. It has been reported that tritium could not be detected in chlorophyll isolated from Chlorella grown in a medium containing tritium oxide (**7**). Considerable research has been done on the effect of deuterium oxide on plant systems. It generally has been reported as retarding respiration (**8**), retarding germination of seeds and spores (**4**), and retarding photosynthesis (**3**) with the degree of retardation increasing with the concentration of deuterium oxide. The concentrations of deuterium oxide employed in these experiments were, however, many orders of magnitude greater than the tracer concentrations of tritium oxide employed in the present study.

Methods

The red kidney bean (*Phaseolus vulgaris*) from a pure strain[2] were used in all experiments. Seeds were sprouted between two dampened pieces of blotting paper in an incubator maintained at 32° C. The seedlings were transferred to glass beads with half-strength nutrient solution to permit establishment of the root systems. After approximately 72 hours in the beads the young plants were placed in culture pans and allowed to grow

[1] This work was performed for the Atomic Energy Commission under Contract no. W-31-109-Eng-52.

[2] California Crop Improvement Association, Davis, California.

until the first trifoliate leaves were one inch across at the base at which time they were ready for experimental use. A modified Hoagland's nutrient solution (**1**) was employed in all experiments, with a pH of 6 except where varied as an experimental condition.

The tritium oxide used in the experiments was produced by burning tritium gas over copper oxide. The tritium oxide was collected in a dry ice trap and diluted with distilled water to give the desired activity in the nutrient solution.

The plants were exposed to tritium oxide in a closed chamber. Air was circulated by a fan through the chamber and over a condenser coil which removed the water of transpiration. Three porcelain refrigerator pans, each holding eight plants supported by lucite holders, served as the nutrient culture containers. Tritium oxide was present in the nutrient solutions at a level of 100 μc./ml. in all experiments. Water of the same tritium oxide content was used to replace the transpired water during the exposure. The light source consisted of twelve 30-watt, cool white fluorescent tubes which gave an average light intensity of 800 f.c. at the pan surface. Diurnal lighting was employed with 16 hours of light per day, except where varied as an experimental condition.

Plants were grown in the tritium oxide solution for 72 hours, duplicate plants being harvested at intervals during this period. Plants remaining at 72 hours were transferred to a tritium oxide-free nutrient solution and sampled at intervals over the remainder of the experimental period.

Plants removed for samples were clipped at the root level and divided into the following tissues: primary leaves, first trifoliate leaves, second trifoliate leaves, and stems. Water was removed from the samples by azeotropic distillation with benzene. The tritium concentration of this plant water was determined by producing acetylene from the water and calcium carbide and measuring the activity of the acetylene with a vibrating reed electrometer. Dry plant tissue, after the above water extraction, was weighed and burned over copper oxide in a microcombustion furnace. The water of combustion was collected in a dry ice trap, converted to hydrogen with calcium metal, and the hydrogen counted in a Geiger tube. Details of the counting procedure will be published by others (**5**).

The apparatus employed for exposing the bean plants to a tritium gas atmosphere is shown in figure 1. The second trifoliate leaf was sealed into the exposure bulb with lanolin and exposed to an atmosphere containing approximately 200 μc. tritium gas/cc. The tritium gas was measured volumetrically by means of a mercury column (a) which compressed the gas into the calibrated glass tube (A) at a constant pressure so equal volumes could be introduced to the mixing chamber (B) by letting a measured volume of gas pass through the stopcock at the top of tube A. After a short time allowed for mixing with the air in chamber B, the atmosphere containing tritium gas was passed into the exposure chamber (c) by filling the mixing chamber with water. The exposure chamber prior to the introduction of the tritium atmosphere was reduced below atmospheric pressure suffi-

ciently to accommodate the gas introduced from chamber B. After an exposure of five hours, the tritium atmosphere was swept from the chamber with oxygen introduced at opening (d) and burned in a microcombustion furnace over copper oxide. The water of combustion was collected in a dry ice trap and the activity determined as a check on the tritium activity of the atmosphere. Plants were sampled and processed as described for the tritium oxide exposures.

Results

The upper group of curves in figure 2 shows the uptake and retention of tritium oxide in the plant water of the tissues studied. After about 12 hours exposure to the nutrient solution containing tritium oxide, the tritium con-

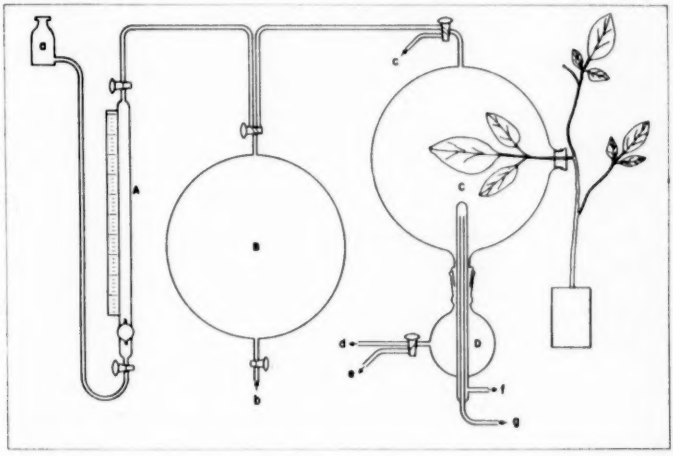

FIG. 1. Apparatus used for exposing bean plants to a tritium gas atmosphere.

centration of all plant water samples reached an apparent plateau equal to 45 to 65% of the tritium concentration of the nutrient solution. In the retention phase of the experiment the water of the various plant parts lost tritium at a rapid rate for several hours, after which further loss of tritium occurred at a much slower rate. The lower group of curves represents similar data for tissue-bound tritium in the same plants. Although plotted to different scales, the two sets of curves in figure 2 are directly comparable. Since the plant tissues were rather uniformly 90% water, the two scales are practically identical.

Figure 3 shows the uptake and retention of tritium in the plant water and total dry tissue as affected by the pH of the nutrient solution. Plants grown in solutions with a pH of 4 tended to gain and lose tritium faster than those grown at pH 7, but the difference was relatively small.

Figure 4 compares the uptake and fixation of tritium from a tritium oxide root environment by plants grown in light intensities of 800 f.c. with

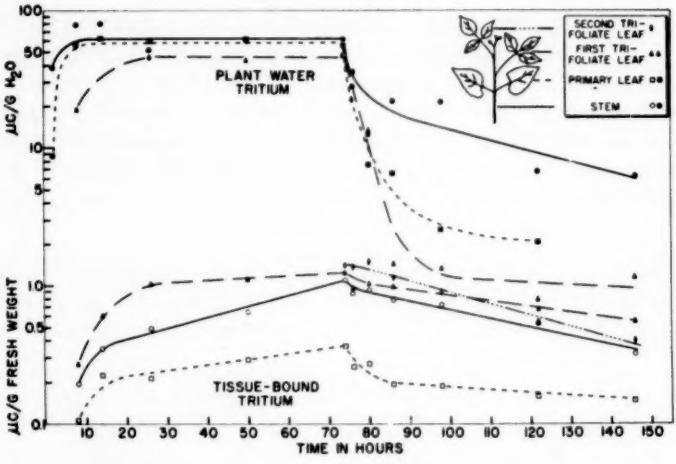

FIG. 2. Uptake and retention of tritium in the plant water and tissue when exposed to tritium oxide root environment. Tritium oxide was present in the nutrient solutions at a level of 100 μc./ml. up to 70 hours at which time no additional tritium was given the plants.

similar data for plants grown in total darkness. Approximately five times more tritium was fixed in the tissues grown in light than in those grown in darkness. The factor for the plant water was approximately 2.5.

Results of the two experiments in which leaves were exposed to tritium gas are shown in table I.

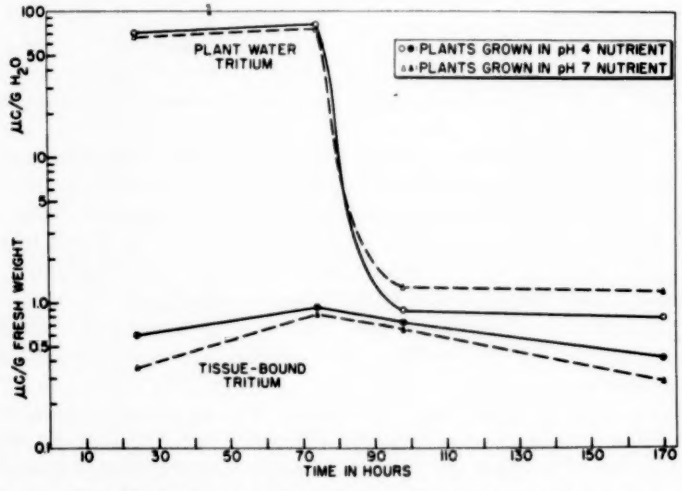

FIG. 3. The pH effect on the uptake and fixation of tritium in the plant water and tissue. Tritium oxide was present in the nutrient solutions at a level of 100 μc./ml. up to 70 hours at which time no additional tritium was given the plants.

Discussion

The failure of the plant water to more closely approach equilibrium with the nutrient solution (with respect to tritium oxide content) was unexpected. This lack of rapid equilibration is evidenced, not only by the uptake curves in figures 2, 3, and 4, but also by the retention curves following exposure which indicate that a small portion of the tritium oxide is rather tenaciously retained. This effect might be due to the slow dilution of colloidally bound water present in the plant tissues. CRAFTS *et al.* (**2**), have indicated that the forces operative in restricting the movement of tissue water will vary from the simple intermolecular forces between the water molecules themselves to intense binding forces found in the hydration of colloids. It would seem reasonable to expect, however, that even this tightly bound water of hydration would be in exchange equilibrium with the rest of the plant water.

TABLE I

TRITIUM IN PLANT TISSUES RESULTING FROM EXPOSURE OF LEAF TO TRITIUM GAS FOR FIVE HOURS.

Plant Tissue	Plant water Tritium (μc./gm.)		Tissue-Bound Tritium (μc./gm. fresh wt.)	
	Run I	Run II	Run I	Run II
Stems and leaves above exposure	560	170	.01	.004
Stems and leaves below exposure	270	40	.002	.002
Exposed leaf	11,000	7,600	.039	.079
Roots	170	50	.015	.017

Note: A total of 88 mc. and 129 mc. of tritium was recovered from the exposure chamber following Runs I and II, respectively.

There remains the possibility that the HTO molecules are not absorbed and translocated at the same rate as the H_2O molecules. The possibilities of such an isotope effect are being further investigated.

The extent of incorporation of tritium into the plant tissues was greatest in the most actively metabolizing tissues, the first and second trifoliate leaves; and lowest in the relatively dormant primary leaves. Similarly the loss of tissue-bound tritium following exposure was most rapid in the younger plant parts and at least rapid in the older primary leaves.

As indicated in figure 3, the pH of the nutrient solution has little effect on tritium oxide uptake and retention or on the incorporation of tritium into plant tissues. The somewhat greater uptake from the more acid solution is perhaps an indication of greater root permeability. Such an effect has been previously observed in the absorption of many mineral elements (**6**).

The light-dark experiments indicate that the major portion of tritium incorporation occurs in the light. This may simply be a reflection of generally increased metabolic activity of the photosynthesizing plant. It is evident that tritium incorporation is not dependent on light although it

appears probable from the greatly increased incorporation in light that photosynthesis is a factor.

The leaf exposures to tritium gas constitute little more than an introduction to the problem of hydrogen fixation by plants, and the results cannot be unequivocally interpreted. Two findings stand out as particularly significant: (1) the great bulk of the tritium fixed is retained in, or on, the exposed leaf, and (2) the relatively very small quantities of tissue-bound tritium present, as compared to plant water tritium. It is apparent that the tritium gas is not extensively distributed throughout the plant, but that most

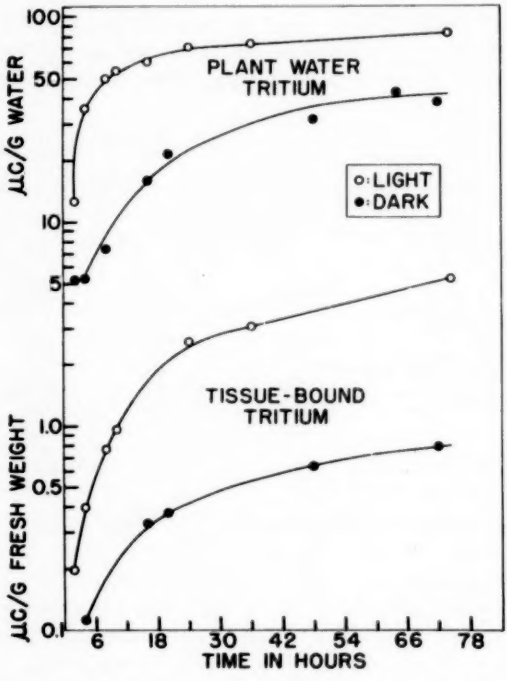

FIG. 4. The uptake and fixation of tritium by the bean plant in darkness. Tritium oxide was present in the nutrient solution at a level of 100 μc./ml.

of the conversion to the oxide must occur in or on the exposed leaf. The very slight degree of incorporation into tissue compounds suggests that the tritium oxide formed in these exposures became involved in the general physiology of the plant to a much lesser degree than was the case with tritium oxide absorbed through the roots. Conversion of the tritium gas to tritium oxide may have occurred largely as a result of exchange of tritium with the protium of water at the leaf surface. It is also possible that tritium gas adsorbed on the leaf surface may to some extent have been converted to the oxide in the course of the analytical procedure involved in separation of plant water. The general problem of the exchange of tritium gas with

ordinary water under the influence of various physiological and non-physiological factors is being further investigated.

Summary

The uptake of tritium oxide by red kidney bean plants grown in a tritium oxide labeled nutrient medium was quite rapid for the first few hours, but equilibrium between plant water and nutrient medium was not attained in the 72-hour experimental period. This lack of complete tritium oxide equilibration may be due to slow dilution of colloidally bound water, or possibly to an isotope effect.

The rate and extent of tritium incorporation in the plant tissue compounds can be correlated directly with the metabolic activity of the tissue concerned.

Absorption of tritium oxide and incorporation of tritium in plant compounds was slightly greater from a nutrient medium of pH 4 than from a nutrient medium of pH 7. Both tritium oxide absorption and tritium incorporation in plant compounds was markedly reduced in plants grown in the dark.

The exposure of a plant leaf to tritium gas resulted in high concentrations of tritium in the plant water of the exposed leaf, much smaller amounts in the plant water from other tissues, and only very slight incorporation in tissue compounds.

The guidance of Dr. J. H. Rediske throughout the course of this study is gratefully acknowledged, as are also the suggestions of Dr. R. C. Thompson. Tritium samples were counted by the Analysis Group of the Biology Section.

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BRIEF PAPERS

ENZYMATIC SYNTHESIS OF GLUTAMINE IN HIGHER PLANTS[1]

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Received December 16, 1952

The synthesis of glutamine has been studied extensively with enzyme preparations from animal tissues and from bacteria (**1, 2, 3, 9, 10**). These studies have shown that in such organisms, glutamine may be formed directly from glutamic acid and ammonia, the energy for the synthesis being supplied as adenosine triphosphate (ATP). Little is known, however, of the occurrence or characteristics of similar reaction systems in higher plants. ELLIOTT (**1**) has reported the formation of glutamyl-hydroxamic acid in homogenates of Lupinus seedlings in the presence of added glutamate, hydroxylamine, ATP, and magnesium ions. This indicates that amide formation in these species probably proceeds in a manner analogous to its formation in other organisms. The present communication concerns studies on the general occurrence of glutamine synthesis in higher plants. Information is also presented on the nature of the synthetic system and its cellular localization.

Homogenates of various seedlings, prepared in the manner described by MILLERD *et al.* (**6**) were dialyzed with shaking for three hours at 1° C against 0.01 M phosphate buffer (pH 7.0). For studies on cellular location of enzyme activity, undialyzed homogenates (from which heavy particles had been removed at 500 × g.) were centrifuged at 10,000 × g. as previously described (**6**) in order to obtain mitochondria. The supernatant from this operation was then centrifuged at 70,000 × g. to obtain the microsomal fraction.

Acetone powders were prepared by grinding the plant material in five volumes of chilled acetone in a Waring Blendor at 1° C. The suspension was filtered with suction at 1° C, and the residue sucked dry in a stream of cold air. The operation was repeated, and the coarse powder was sifted through a screen. The resulting powder was stored in a desiccator at 1° C. Active extracts of acetone powders were prepared essentially in the manner described by SPECK (**10**).

Unless otherwise indicated, the complete reaction system contained: 0.05 M potassium phosphate buffer (pH 7.0), 0.05 M sodium L-glutamate, 0.002 M sodium adenosine triphosphate, 0.02 M $MgSO_4$, 0.04 M NH_4Cl, 0.01 M cysteine, and one ml. of enzyme preparation in a total volume of

[1] This investigation was supported in part by the Polychemicals Department, E. I. du Pont de Nemours and Co.

TABLE I

GLUTAMINE SYNTHESIS BY HOMOGENATES OF VARIOUS PLANT TISSUES.

Family	Experimental material	Glutamine formed (μ M/ml. enzyme)	Glutamyl-hydroxamic acid formed (μ M/ml. enzyme)
Leguminosae	Bean seedling	2.15	2.53
	Lupine seedling	2.13	2.23
Solanaceae	Potato tuber	1.05	1.31
Compositae	Sunflower root	1.51	1.32
Chenopodiaceae	Beet root	2.70	2.95
Gramineae	Corn seedling	1.07	1.15
	Wheat germ	2.05	2.20
Umbelliferae	Carrot root	1.58	1.33
Convolvulaceae	Sweet potato root	1.16	1.54

4 ml. Controls contained one ml. of enzyme preparation which had been boiled for five minutes. The reaction mixtures were shaken at 25° C for 30 minutes. The course of the reaction was then stopped by the addition of cold acetone, and the precipitated protein removed by centrifugation at 5000 × g. The acetone was next removed under vacuum. Amide formation was measured by a new and convenient method. Measured quantities (usually 0.3 ml.) of the concentrated reaction mixture were applied across one side of a sheet of Whatman No. 1 filter paper, and the components separated by descending chromatography with water-saturated phenol. The faster-moving glutamine was easily separated from glutamate, and was eluted from the paper by the procedure of LANDUA and AWAPARA (**4**). The glutamine concentration was determined with ninhydrin by the method of MOORE and STEIN (**7**). After glutamine formation was established by this method, NH_4Cl was replaced by NH_2OH in the reaction system, and the more convenient estimation of glutamyl-hydroxamic acid was made by the method of LIPMANN and TUTTLE (**5**). Hydrolysis of ATP was determined by incubating 0.5 ml. of

TABLE II

SYNTHESIS OF GLUTAMINE BY EXTRACTS OF BEAN SEEDLINGS.

System	Hydroxamic acid formed (μ M/ml. enzyme)	Inorganic phosphate formed (μ M/ml. enzyme)
Complete	1.27	1.31
Without glutamate	0.28	0.25
Without NH_2OH	0.00	0.09
Without ATP	0.07	0.00
Without $MgSO_4$	0.19	0.18
Without cysteine	0.64	0.75
Plus coenzyme A	1.21	1.28
Plus yeast extract	1.29	1.35

Complete system contained: 0.03 M tris-(hydroxymethyl)-aminomethane buffer (pH 7.5), 0.05 M glutamate, 0.002 M ATP, 0.02 M $MgSO_4$, 0.04 M NH_2OH, 0.01 M cysteine, and one ml. of an enzyme preparation (**10**) from bean seedling acetone powder. A similar preparation in which NH_4Cl was substituted for NH_2OH formed 1.03 micromoles of glutamine under the same conditions.

enzyme preparation with 0.3 ml. of 0.01 M tris-(hydroxymethyl)-aminomethane buffer (pH 7.4) containing 0.001 M $CaCl_2$, and 0.2 ml. of 0.01 M ATP for 30 min. at 25° C. Enzymatic liberation of inorganic phosphate was used as the measure of ATP hydrolysis.

Table I summarizes findings on the formation of glutamine and glutamyl-hydroxamic acid by homogenates from various sources. It is evident from the data that the enzyme system responsible for glutamine synthesis is widely distributed among higher plants.

That the simultaneous presence of glutamate, ATP, Mg ions, and NH_3 or NH_2OH is essential for the synthetic reaction is clearly indicated in table II. Cysteine, although it definitely increases the rate of the reaction, is not essential and furthermore can be replaced by other reducing agents. It is also evident from the data of table II that the liberation of inorganic phosphate parallels the formation of amide. The overall synthetic reaction in plants, therefore, probably proceeds in the same manner as has been established in animal tissues, namely:

$$\text{Glutamate} + NH_3 + \text{ATP} \xrightarrow{Mg^{++}} \text{Glutamine} + \text{ADP} + \text{inorganic phosphate}$$

No necessity for co-factors was readily apparent. Well-dialyzed preparations were completely active using the reaction system described above. The addition of either coenzyme A or an extract of boiled yeast caused no significant increases in activity.

Cellular localization of the synthetic activity is of particular interest since FREI and LEUTHARDT (3) were unable to definitely locate glutamine synthesis in any one of the cellular fractions of liver homogenates. They reported instead that a combination of the mitochondrial and soluble fractions was necessary for maximal activity. This is not the case with bean homogenates. Using homogenates prepared in 0.05 M potassium phosphate buffer (pH 7.3)—0.45 M sucrose in which the mitochondrial organization is largely preserved, it is possible to demonstrate that a large proportion of the total activity of the homogenate resides in the mitochondrial fraction (table III). Recombination of the various fractions resulted in no significant increase in activity above that expected from their individual activities. The possibility was considered that the presence of large amounts of ATP

TABLE III

CELLULAR LOCALIZATION OF GLUTAMINE SYNTHESIS IN BEAN SEEDLING HOMOGENATES.

Fraction	Sedimentation		Fraction (per cent.) of total	
	Time min.	Acceleration g.	Glutamine synthesis	ATP hydrolysis
Nuclear	10	500	0	16
Mitochondrial	30	10,000	61	37
Microsomal	60	70,000	21	19
Soluble	....	....	18	28

hydrolysis activity in one or more of the cellular fractions might be influencing the results by lowering the ATP level significantly. As can be seen from table III, however, the hydrolysis of ATP by the various cellular fractions roughly parallels the activity for the synthesis of glutamine. ATP hydrolysis does not appear, therefore, to influence importantly the results on the distribution of synthetic activity.

In the case of homogenates prepared by grinding the seedlings in a Waring Blendor with an equal volume of 0.1 M potassium phosphate buffer (pH 7.1), up to 95% of the synthetic activity can be found in the soluble fraction. It would seem, therefore, that the enzyme system responsible for glutamine synthesis may be largely (or entirely) associated with the mitochondria in the intact bean cell, but is bound by a somewhat labile linkage. A similar situation has been reported in a study of the hexokinase of potato tubers (**8**). The improved methods now available for the isolation of plant mitochondria are thus making it possible to obtain more insight into the localization of this and other synthetic enzymes.

The author wishes to thank Dr. James Bonner for his helpful comments during this investigation.

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ENZYMATIC SYNTHESIS OF GAMMA-GLUTAMYL-CYSTEINE IN HIGHER PLANTS[1]

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Received January 28, 1953

It has been demonstrated that enzyme preparations from higher plants catalyze the following syntheses in the presence of adenosine triphosphate (ATP) and magnesium ions (**2, 8**):

$$\text{Glutamate} + NH_3 = \text{Glutamine}$$
$$\text{Glutamate} + NH_2OH = \text{Glutamyl-hydroxamic acid}$$

It therefore seemed possible that the synthesis of dipeptides involving the glutamyl moiety might occur by an analogous reaction. The glutamyl peptide, gamma-glutamyl-cysteinyl-glycine (glutathione), is known to occur generally in plants. The enzymatic synthesis of gamma-glutamyl-cysteine has therefore been first investigated since this compound constitutes a possible intermediate in the synthesis of glutathione. JOHNSTON and BLOCH (**4**) have already demonstrated a necessity of ATP for glutathione formation by liver preparations, and SNOKE and ROTHMAN (**7**) have reported the condensation of gamma-glutamyl-cysteine and glycine to form glutathione in the same system.

The enzyme system used in this work consisted of dialyzed extracts of acetone powders of bean (*Phaseolus vulgaris*) seedlings prepared as previously described (**8**). The enzyme was incubated for 60 minutes at 25° C in a reaction mixture consisting of: 0.05 M phosphate buffer (pH 7.0), 0.01 M glutamic acid-2-C^{14} (specific activity—0.1 mc./mM), 0.01 M cysteine, 0.002 M ATP, 0.005 M $MgSO_4$, and 0.005 M KCl in a total volume of 5 ml. The reaction was stopped by the addition of cold acetone, and the precipitated protein removed by centrifugation at $5000 \times g$. The supernatant solution was evaporated to dryness under a vacuum, and the residue was taken up in a small quantity of a solution of *N*-ethyl maleimide as described by HANES *et al.* (**3**). The components present in the solution were next separated by paper chromatography (**8**). Best separation was obtained with a butanol-acetic acid-water mixture (4 : 1 : 1) as the solvent. The presence of radioactive material on the chromatogram was indicated by radioautography (**1**), while non-radioactive amino acids and peptides were detected with ninhydrin. Table I gives R_f values obtained in a typical experiment. It is evident that a radioactive substance has been formed enzymatically which has an R_f identical to that of authentic gamma-glutamyl-cysteine prepared synthetically as described elsewhere (**9**). Further-

[1] This investigation was supported in part by the Polychemicals Department, E. I. du Pont de Nemours and Co.

TABLE I

RESULTS OF CHROMATOGRAPHIC SEPARATION OF THE COMPONENTS INVOLVED IN THE SYNTHESIS AND IDENTIFICATION OF GAMMA-GLUTAMYL-CYSTEINE.

Component	R_f			
	0.05	0.33	0.45	0.53
Cystine alone	N			
Glutamate alone		N		
Cysteine alone				N
Gamma-glutamyl-cysteine alone			N	
Glutamate-C^{14} + cysteine + boiled enzyme preparation		R		N
Glutamate-C^{14} + cysteine + active enzyme preparation		R	R	N
Products of acid hydrolysis of enzymatically formed peptide	N	R		
Products of acid hydrolysis of authentic gamma-glutamyl-cysteine	N	N		

All spots on the chromatogram corresponded to the above four R_f values. Values given for cysteine refer to its ninhydrin-reactive derivative with *N*-ethyl maleimide. Non-radioactive spots indicated by N; radioactive spots indicated by R.

more, when a portion of the reaction mixture was subjected to acid hydrolysis before chromatography, only glutamic acid and cystine were recovered (table I). Hydrolysis of a sample of authentic gamma-glutamyl-cysteine gave the same result. That the substance formed was glutamyl-cysteine rather than cysteinyl-glutamate, was indicated by treating a sample of the peptide (isolated by precipitation as the cadmium salt as described elsewhere (**9**)) with 2,4-dinitrofluorobenzene (**5**), and subjecting the resulting *N*-dinitrophenylpeptide to acid hydrolysis. Chromatography of the products of hydrolysis with tertiary amyl alcohol showed only *N*-dinitrophenyl-glutamate to be present, thus demonstrating that the amino position of the glutamyl residue is free in the original peptide. That the linkage was gamma, rather than alpha, was indicated by its ease of hydrolysis in dilute acid. It may, therefore, be concluded that the product of the enzymatic reaction studied here is gamma-glutamyl-cysteine.

A preliminary examination was made of the nature of the synthetic reaction. Quantitative estimation of the formation of glutamyl-cysteine

TABLE II

SYNTHESIS OF GAMMA-GLUTAMYL-CYSTEINE BY EXTRACTS OF ACETONE POWDERS OF BEAN SEEDLINGS.

System	Glutamyl-cysteine formed (μg./ml. enzyme)
Complete	141
No glutamate	4
No cysteine	5
No ATP	9
No ATP–adenosine monophosphate	10
No magnesium ion	17
No potassium ion	65
Plus coenzyme A	143

was made by radioactive assay (**9**). No evidence for a glutamyl exchange reaction (**9**) was found during the formation of glutamyl-cysteine. The data of table II indicate the degree to which the various components of the reaction system are essential to the progress of the reaction. It is evident that the presence of ATP and magnesium ions is necessary for the formation of the peptide, and ATP cannot be replaced by adenosine monophosphate. The presence of potassium ion also appears to be necessary for maximal activity of the enzyme system. That an absolute requirement for metal ions is not shown is probably due to our inability to completely free the enzyme protein of metals by dialysis. The addition of coenzyme A did not influence the course of the reaction under the present conditions, a characteristic also found for glutamine synthesis by plant systems (**8**). It appears probable then, that the formation of glutamyl-cysteine proceeds in a manner similar to the synthesis of glutamine as previously reported (**2, 8**).

As this investigation was being completed, SNOKE and BLOCH (**6**) reported a very similar enzymatic synthesis of gamma-glutamyl-cysteine by preparations from mammalian liver. It appears, therefore, that the formation of this peptide may be a feature common to various organisms.

The author wishes to thank Dr. James Bonner for his helpful comments during this investigation.

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THE BIOSYNTHESIS OF CARBON-14-LABELED COMPOUNDS. III. THE SEPARATION AND ISOLATION OF SUGARS BY ION-EXCHANGE CHROMATOGRAPHY[1]

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Received December 16, 1952

Introduction

One of the major difficulties in the preparation of labeled compounds by biosynthetic procedures is the isolation of chemically and radiochemically pure compounds. Often compounds which appear chemically pure are found to contain radioactive contaminants which may make such labeled compounds virtually useless in biological experiments. UDENFRIEND and GIBBS (**11**) and more recently PUTMAN and HASSID (**8**) used paper chromatography to effect the separation and isolation of several labeled sugars. The latter workers pointed out that such a procedure enabled them to prepare sugars of very high specific activity since it was unnecessary to add any carriers. Furthermore, they were able to obtain labeled sugars containing extremely small amounts of radioactive contaminants. NOGGLE and BOLOMEY (**5**) used column chromatography to effect the separation of glucose and fructose while GIBBS *et al.* (**2**) prepared labeled sucrose by fermenting out the monosaccharides. NOGGLE and SCHUMACHER (**6**) separated the sugars present in plant extracts into a monosaccharide, a disaccharide, and a trisaccharide fraction by column chromatography on charcoal. Certain disadvantages are found in all of these methods, such as limited capacity, inability to obtain radiochemically pure products, or the necessity of adding carrier, so that it seemed desirable to explore other methods that would minimize these difficulties. It has been known for a long time that sugars and certain other polyhydroxy compounds react readily with borate ions to form borate complexes which are negatively charged ions. KHYM and ZILL (**4**) demonstrated that a mixture of these sugar-borate complexes could be resolved by ion-exchange chromatography on strong-base anion exchange resins. Subsequently, NOGGLE and ZILL (**7**) showed that this procedure could be used for the quantitative analysis of the sugars found in plant extracts and when this method was tried as a preparative method for separating labeled sugars prepared biosynthetically, it was found to give results that overcame many of the difficulties encountered in other preparative procedures. The experimental results are presented in this paper.

[1] Work performed under contract no. W-7405-eng-26 for the Atomic Energy Commission.

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Apparatus

PHOTOSYNTHESIS CHAMBER.—The chamber described by NOGGLE and BOLOMEY (**5**) was used.

ION-EXCHANGE COLUMNS.—The columns have been described elsewhere (**9, 10**). They were 0.85 sq. cm. in cross sections and 18.0 cm. long.

ION-EXCHANGE RESIN.—Dowex-1 strong-base anion exchange resin (Dow Chemical Company, Midland, Michigan) was used. The preparation of the resin has been described (**4**).

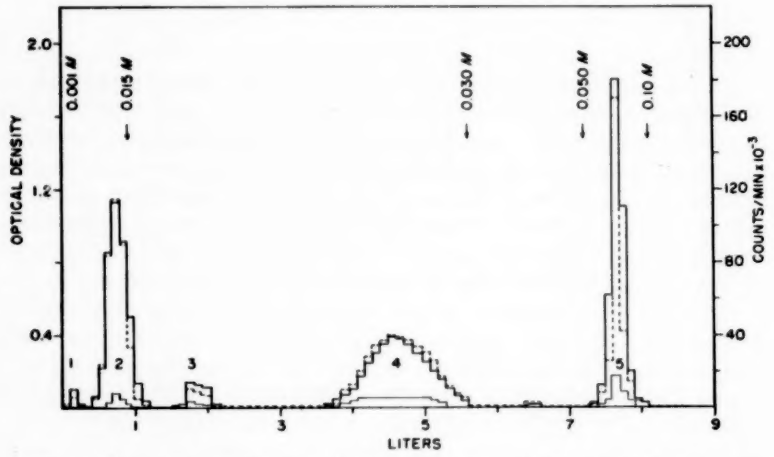

FIG. 1. Separation of the sugars in *Phytolacca decandra* by ion-exchange chromatography of their borate complexes. Resin bed, 0.85 sq. cm. × 12 cm.; Dowex-1 resin (200–400 mesh), borate form. Elution started with 0.001 M potassium tetraborate, changed to 0.015 M at 900 ml., to 0.030 M at 5600 ml., to 0.050 M at 7200 ml., and to 0.10 M at 8100 ml.

——— — optical density (at 620 mμ) with anthrone reagent.
——— — optical density (at 620 mμ) with orcinol reagent.
- - - - - - — radioactivity of 1-ml. aliquots.

Procedure

The leaves of pokeweed (*Phytolacca decandra*) were removed from field-grown plants and placed in the dark for 40 hours. The leaves (70 gm. fresh weight) were then exposed to 1.0 mc. of C^{14} as $C^{14}O_2$ in the photosynthesis chamber for 20 hours at a light intensity of approximately 700 f.c. At the conclusion of the illumination period, the leaves were cut up and dropped into boiling 80% ethanol for 30 minutes. The alcohol was decanted from the leaves, fresh boiling 80% ethanol was added, and the leaves were then macerated in a Waring Blendor for 10 minutes. The macerate was filtered and washed with hot ethanol. The alcohol filtrates were combined, and the alcohol was removed under reduced pressure after which the solution was deproteinized and deionized (**5**) and made up to a known volume.

Preliminary tests indicated that the solution contained approximately 200 mg. of sugar. Since the capacity of the ion-exchange resin bed (0.85 sq. cm. × 12.6 cm.) was approximately 60 mg. of sugar, a 20-ml. aliquot equivalent to roughly 40 mg. of total sugar was separated by the procedure of KHYM and ZILL (**4**). The results are shown in figure 1. Each collected fraction was analyzed for sugar with the anthrone reagent and with orcinol (**7**), and for radioactivity by plating aliquots on aluminum planchets and counting in a gas-flow proportional counter. The first peak through the column was not identified. It is suspected of being a saponin but additional tests must be made. Peaks 2, 3, 4, and 5 were found to be sucrose, raffinose, fructose, and glucose, respectively. The actual amounts, as measured by the optical densities of their anthrone derivatives, were 12.3 mg. of sucrose, 2.1 mg. of raffinose, 13.4 mg. of fructose, and 16.9 mg. of glucose, or a total of 44.7 mg. of sugar in the 20-ml. aliquot. The coincidence of the sugar concentration and radioactivity in each peak suggests that the individual sugars were radiochemically pure. When the isolated sugars were chromatographed and autoradiographs were prepared, no radioactive contaminants could be found.

The separated sugars were isolated by pooling the individual fractions containing a single sugar. Each pooled sample then contained one sugar in a solution of potassium tetraborate which was used to effect the separation. The solutions were treated with a strong-acid cation exchange resin (Dowex-50) in the hydrogen form to remove the potassium ions. The resin was then removed from the solution by filtering through a sintered-glass filter. It is imperative that the potassium ions be removed quickly from the sugar sample because the potassium tetraborate solution used to effect the separation is alkaline and prolonged standing of the sugars in alkaline solutions results in a considerable breakdown. Following removal of the potassium ions the sugar was in a solution of boric acid. The solution was concentrated under reduced pressure (water pump) at 30° C until the boric acid started to crystallize. Absolute methyl alcohol was added, and the volatile methyl borate formed was removed under reduced pressure at 30° C. This step was repeated three times after which practically all the boric acid had been removed. ZILL, KHYM, and CHENIAE (**12**) have shown that 5 to 7

TABLE I

AMOUNT, SPECIFIC ACTIVITY, AND TOTAL ACTIVITY OF SUGARS EXTRACTED FROM POKEWEED LEAVES.*

Sugar	Amount	Specific activity	Total activity
	mg.	*μc./mg.*	*μc.*
Sucrose	61.5	1.89	116.2
Raffinose	10.8	0.96	10.4
Fructose	67.1	1.78	119.4
Glucose	84.7	1.27	107.7

*Pokeweed leaves, 70 gm. fresh weight; had been administered 1.0 mc. of C^{14} as $C^{14}O_2$.

p.p.m. of boric acid remains in the sugar solutions after such treatments with methyl alcohol. The sugars may then be crystallized out by conventional techniques. Some sugar is lost during the manipulations necessary to free the solutions of potassium ions and boric acid, but recovery experiments indicated that approximately 90% of added sugar could be recovered.

In the present study, the sugars were not crystallized since they were to be used in solution for another experiment. Aliquots of the individual sugars were burned, and the CO_2 converted to $BaCO_3$ for counting. The specific activities of the sugars are shown in table I.

Discussion

It can readily be seen that the ion-exchange chromatography method is capable of separating the borate complexes of a great many different sugars. Not only sugars but any compound capable of forming a borate complex may be separated. Sugar alcohols (**12**) and glycosides (**1**) as well as phosphorylated carbohydrates (**3**) have been separated and additional work is under way on other compounds. It is not always possible to predict the conditions necessary to effect a complete separation of the sugars present in a sample of biological origin, but preliminary tests by paper chromatography are usually sufficient to indicate the necessary steps.

The resin beds used in the present experiments were capable of handling 50 to 60 mg. of sugar without having the peaks run together. Larger columns can be used to handle larger amounts of sugar. The sugars can be obtained carrier-free by this method. Since sugars of high specific activity are not generally needed for biological experiments, carrier sugar can be added following removal of the potassium ions and before the removal of the boric acid.

Summary

Carbon-14-labeled sucrose, raffinose, fructose, and glucose were separated and isolated from plant extracts by ion-exchange chromatography of their borate complexes. The sugars were prepared carrier-free and free of chemical and radiochemical impurities as indicated by paper chromatography and autoradiography.

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THE USE OF CATION-EXCHANGE RESINS FOR THE HYDROLYSIS OF SUCROSE IN PLANT EXTRACTS[1]

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Received January 23. 1953

There are a number of reports in the literature regarding the use of ion-exchange materials for the catalytic hydrolysis of esters, proteins, and sucrose (**1, 2, 3, 6, 7**). The work of BODAMER and KUNIN (**1**) on the hydrolysis of sucrose by several readily available synthetic cation exchangers suggested that it might be possible to use this method for the determination of the total sugars in plant samples. The present paper is concerned with some comparative studies on the hydrolysis of sucrose in plant extracts by a cation-exchange resin, acid, and invertase.

Methods

In preliminary experiments, a number of synthetic cation-exchange resins were tested regarding their ability to hydrolyze sucrose. Of these, Dowex-50, Amberlite IR-100, Amberlite IR-120, Amberlite IRC-50, and Duolite C-3,[3] only the Amberlite IRC-50 proved unsatisfactory. In the work that is reported here the Dowex-50 resin was used. It was received in the sodium form (200–400 mesh size) and was converted to the hydrogen form with 5% HCl after which it was exhaustively washed with distilled water. The resin was air dried, using a sintered-glass funnel, and known amounts weighed out for the hydrolysis experiments.

The plant samples were extracted with boiling 80% ethanol for 30 minutes. The alcohol was decanted off and the plant material was extracted in a Waring Blendor with hot 80% ethanol and filtered while hot. The alcohol filtrates were combined and concentrated under reduced pressure to remove the alcohol.

The alcohol-free extracts were filtered through Celite Analytical Filter-Aid (**8**) and then clarified with the aid of mixed anion- and cation-exchange resins (**9**). The resin was a Monobed preparation of Amberlite IR-120 and Amberlite IR-4B. Reducing sugars were determined by the

[1] Work performed under contract no. W-7405-eng-26 for the Atomic Energy Commission.

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[3] The Dowex-50 resin was obtained from the Dow Chemical Company, Midland, Michigan, the Amberlite resins from the Rohm & Haas Company, Philadelphia, Pennsylvania and the Duolite C-3 resin from the Chemical Process Company, Redwood City, California.

use of the modified Somogyi reagent (**5**). Total sugars were determined by the Somogyi method following either invertase or HCl hydrolysis (**4**). A 10-ml. aliquot of the cleared extract was heated with 10 ml. of HCl (sp. gr. 1.1) for 11 minutes in a water bath at 70° C. After cooling for 30 min., the solution was neutralized with 20% sodium hydroxide, made to volume, and analyzed for reducing sugar. For the invertase hydrolysis, 5 ml. of the cleared extract were added to 5 ml. of 0.2 M acetate buffer (pH 4.5) and 1 ml. of 0.2% invertase solution (Nutritional Biochemicals Corp.). The hydrolysis was carried out overnight, after which the mixture was neutralized with 0.1 *N* NaOH, made to volume, and analyzed for reducing sugar. Pure sucrose was also carried through the above procedures.

Results

PURE SUGARS AND POLYSACCHARIDES.—Preliminary experiments indicated that sucrose could be completely hydrolyzed by the following procedure: The sucrose, 5 to 250 mg. dissolved in 5 ml. of water, was mixed with 1 gm. (moist weight) of Dowex-50 in a test tube that was immersed in a boiling water bath. After 40 minutes of continuous stirring the solution was cooled

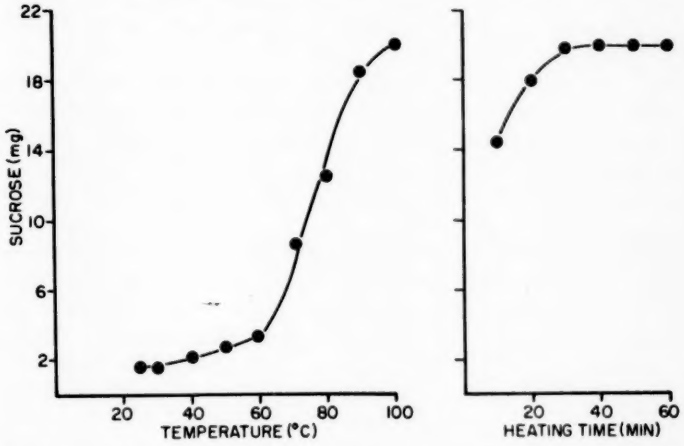

FIG. 1 (*left*). Sucrose hydrolysis at different temperatures. 20 mg. sucrose hydrolyzed with 1 gm. of Dowex-50 at different temperatures. Solutions stirred for 40 minutes.

FIG. 2 (*right*). Rate of sucrose hydrolysis at 100° C. 20 mg. sucrose hydrolyzed with 1 gm. Dowex-50 for different periods of time.

and filtered through a sintered-glass filter. The effect of varying the temperature of the water bath or of the time of the hydrolysis is shown in figures 1 and 2.

A series of oligosaccharides was treated with Dowex-50 under the conditions that completely hydrolyzed sucrose. The sugar solutions were paper chromatographed using the techniques followed by WILLIAMS and

TABLE I

PERCENTAGES OF REDUCING SUGAR AND TOTAL SUGAR (EACH AS GLUCOSE) AFTER ACID HYDROLYSIS OR DOWEX-50 HYDROLYSIS OF EXTRACTS OF TOMATO, TOBACCO, AND BARLEY LEAVES. BASED ON FRESH WEIGHT OF LEAVES.

Plant	Reducing sugar	Reducing sugar after	
		HCl hydrolysis	Dowex-50 hydrolysis
	%	%	%
Tobacco	0.027	0.080	0.085
Tomato	0.078	0.13	0.13
Barley	0.046	0.33	0.31

BEVENUE (8). Melibiose, maltose, cellobiose, and turanose were not hydrolyzed, while raffinose and melezitose were partially hydrolyzed.

Starch and inulin were treated with Dowex-50 and separately with HCl under conditions that would completely hydrolyze sucrose. The filtrates, after removal of the resin or neutralization of the HCl, were analyzed for reducing sugars. No reducing sugars were found from either the resin- or HCl-hydrolyzed starch samples. The HCl-hydrolyzed inulin sample titrated completely as reducing sugar, while from the resin hydrolysis only a trace of reducing sugar could be found.

PLANT EXTRACTS.—Twenty-five gm. (fresh weight) of leaves from greenhouse-grown tomatoes, tobacco, and barley were extracted and clarified. Reducing and total sugars (each calculated as glucose) after either acid hydrolysis or Dowex-50 hydrolysis were determined on the plant extracts. The results are shown in table I, where it is apparent that the two methods gave very similar results.

Another plant sample was extracted with alcohol and then clarified. Reducing sugars after acid hydrolysis, invertase hydrolysis, and Dowex-50 hydrolysis were determined. The values were as follows (measured as mg. glucose per aliquot): acid hydrolysis, 70.5 mg.; invertase hydrolysis, 69.2 mg.; and Dowex-50 hydrolysis, 71.8 mg. The values agree very well and indicate the usefulness of the Dowex-50 hydrolysis method for determining total sugars in plant extracts. This is true provided that sucrose is the only oligosaccharide present. If raffinose is a major constituent, acid must be used, since the Dowex-50 treatment only partially hydrolyzes raffinose.

TABLE II

RECOVERY OF ADDED SUCROSE FROM A PLANT EXTRACT.

Plant extract	Added sucrose	Reducing sugar found	Recovery
ml.	*mg.*	*mg.*	%
1	None	1.8	....
1	10	11.7	99
1	25	27.0	101
1	50	51.4	99

RECOVERY EXPERIMENTS.—A series of aliquots of a plant extract to which had been added variable amounts of sucrose were hydrolyzed by Dowex-50. The filtrates were analyzed for reducing sugar and the results as shown in table II indicate that the recoveries are essentially quantitative.

Discussion

Sucrose was readily hydrolyzed by the Dowex-50, a sulfonic acid cation-exchange resin. BODAMER and KUNIN (**1**) similarly noted that the sulfonic acid resins caused rapid sucrose inversion as opposed to the carboxylic type resins. They also suggested that the rate of sucrose inversion was a function of the resin particle size and degree of porosity. In the present work a fine-mesh resin was used in connection with constant stirring, so that in all probability the maximum contact between sugar and resin was obtained.

The results from the hydrolysis of sucrose in plants indicate that the cation-exchange resin hydrolysis is as effective as either the acid hydrolysis or invertase hydrolysis. The method requires no special equipment: the resin is stable and can be used over again. When compared with the acid methods, the resin hydrolysis is considerably easier. There is no excess acid to neutralize and volumes can be kept small. It is very often found that the acid method hydrolyzes non-sugar materials, such as dextrines or inulin, which are subsequently determined as reducing sugar. With the resin method, the hydrolysis of such compounds is very small.

The resin hydrolysis is quicker than the invertase method, and since the resin is stable, there is no problem of maintaining a labile enzyme preparation on hand.

The combination of the batch-resin clarification technique of WILLIAMS and BEVENUE (**9**) with the resin hydrolysis of sucrose appears to offer a number of advantages in the analysis of the sugar in plant extracts. The resin clarification seems to remove non-sugar reducing materials from the plant extracts that are not removed by such conventional methods as lead phosphate or lead oxalate. It was noted in these experiments that plant extracts cleared by these latter procedures were yellow in color, whereas the batch-resin cleared extracts were water clear. Not only is it an advantage to have the solutions clear when measuring reducing sugar by titration method (**8**) but, according to ROBERTS (**4**), the coloring matter in conventionally cleared extracts may yield quite erroneous results in terms of reducing sugars and total sugars.

Summary

1. Sucrose was completely hydrolyzed by a sulfonic acid-type cation-exchange resin.

2. The resin hydrolysis method was compared with acid and invertase hydrolysis methods for determining the total sugar concentration of plant extracts. All three methods gave comparable results.

3. There are a number of advantages in the use of the cation-exchange resin hydrolysis method that may make it useful to the routine analysis of plant extracts.

I wish to thank Miss Eleanor Schumacher for technical assistance in these experiments.

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CONVERSION OF C^{14}-LABELED ACETATE TO CITRIC AND MALIC ACIDS IN THE TOMATO FRUIT[1]

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Received October 11, 1952

The possible intermediary position of the plant acids in respiration and carbohydrate metabolism as indicated for some plant tissues, suggests that a similar situation may exist in fruit. Many fruits contain comparatively large amounts of various acids (**8**) and apparently have a very active acid metabolism, as shown by the changes in concentration which occur during growth, ripening and storage. TURNER (**10**), for example, found that most of the citric acid in apple tissue was actively engaged in the metabolic flux during the post-harvest cold storage period. He concluded that the data of his experiments are compatible with the participation of a Krebs cycle in carbohydrate oxidation in the apple, but do not prove that such a cycle exists.

While C^{14}-labeled substrates have been used extensively for studying intermediary metabolism in some plant tissues, their use in connection with fruit has not been reported, so far as known. In preliminary experiments with tomato fruit, it was found that following injection with C^{14}-labeled sodium acetate, radioactivity appeared in the respiratory CO_2, the acids, and in certain other fractions isolated from the juice and tissue. Data from degradation studies on citric and malic acid which suggest the participation of a Krebs cycle in tomato fruit metabolism are presented.

Experimental

The fruits used in this experiment were greenhouse grown Michigan State Forcing tomatoes picked at the mature green state. Samples were collected in the evening and kept in a refrigerator at 3° C until 7:30 A.M. when treatments were started. Carboxyl C^{14}-labeled sodium acetate with a specific activity of 3.00×10^8 counts per minute per mM (c.p.m.) was prepared by the conventional Grignard reaction and dissolved in water to provide a concentration of 1 mg./ml. By means of a hypodermic syringe, 6 ml. of this solution were injected into the locules of two tomatoes weighing 284 and 199 gm., respectively. Preliminary experiments with dye solutions showed that solutions injected by this method were absorbed mainly by the locular tissue and to a lesser extent by the pericarp. The treated samples

[1] Published with approval of the Monographs Publications Committee, Oregon State College. Research paper no. 223, School of Science, Department of Chemistry. This research was supported by contract No. AT (45-1)-573 from the Atomic Energy Commission.

were transferred immediately to a desiccator at 28° C and kept at this temperature during the course of the experiment.

The evolved CO_2 was aspirated from the chamber with CO_2-free air and collected in sodium hydroxide solution contained in a gas washing bottle fitted with a sintered glass disperser. The solution was replaced every four hours and the absorbed CO_2 was precipitated as barium carbonate for radioactivity assay. The specific activity of the respiratory CO_2 reached a maximum in approximately 14 hours and declined thereafter. Meanwhile the rate of increase in the cumulative radioactivity began to level off in approximately 28 hours, indicating that the maximum rate of incorporation had

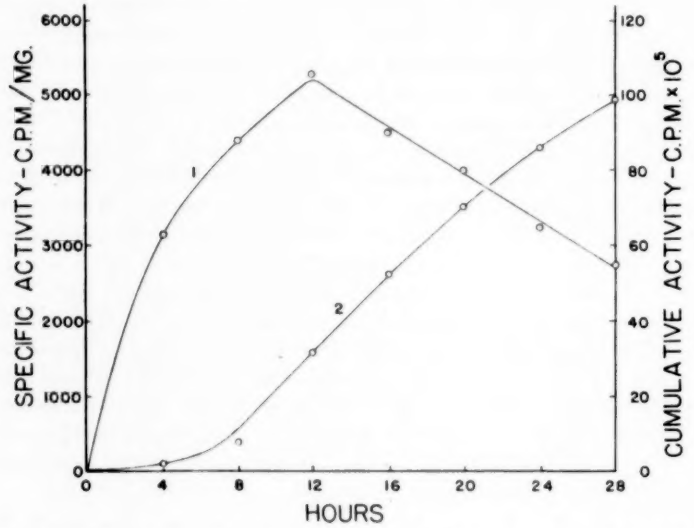

FIG. 1. Radioactivity of respiratory CO_2 (as $BaCO_3$) of tomato fruit following injection with C^{14} carboxyl labeled sodium acetate. 1—specific activity; 2—cumulative activity.

been reached and that further incubation would probably reduce the net incorporation of the labeled substrate in the fruits.

The fruits, therefore, were transferred to a blendor and finely ground. The juice was separated by centrifuging and adjusted to pH 3 with sulfuric acid. After standing overnight the precipitated protein was removed by centrifuging. The clear juice thus obtained was further acidified with sulfuric acid and extracted with ether for 72 hours in a liquid-liquid extractor. The ether extract was evaporated to dryness under vacuum, the residue was taken up in 10 ml. distilled water, and after being chilled in a refrigerator the insoluble fatty material was separated by filtration. By titration it was found that the filtrate contained 11.7 mE total acids. The titrated solution was counted by direct plating and was found to have 2.18×10^6 c.p.m., which

represented 10% of the total input and 18% of the radioactivity incorporated. By chemical analyses for citric (**7**) and malic (**6**) acids, the organic acid fraction was found to contain 398 mg. (6.2 mE) of citric acid and 354 mg. (5.3 mE) of malic acid. The close agreement of the total milliequivalents of acids found by titration (11.7) and by analysis for citric and malic acids (11.5) indicate that only traces of other acids could have been present.

To determine the incorporation and distribution of C^{14} in citric and malic acids, one-half of the acid solution was neutralized with sodium hydroxide, evaporated to dryness under vacuum, and the hydroxy acids separated by means of partition chromatography according to the method of BULEN, VARNER, and BURRELL (**1**). Only citric and malic acids could be identified positively on the chromatogram. The citric acid fraction was concentrated to 25 ml. and was found by analysis to contain 2.51 mg./ml. Similarly, the malic acid fraction concentrated to 10 ml. was found to contain 5.85 mg./ml.

Each of the pure acids thus obtained was diluted with non-radioactive carrier, acidified with sulfuric acid and extracted with ether in a liquid-liquid extractor. The ether extracts were dried over sodium sulfate and evaporated. Citric acid was degraded according to WEINHOUSE, MODES, and FLOYD (**11**). Malic acid was degraded according to WOOD, WERKMAN, HEMMINGWAY, and NIER (**12**).

All samples were counted as barium carbonate to a standard error of two per cent. and corrected for background and self-absorption.

The protein fraction isolated from the juice was combined with the protein obtained from the pulp by sodium borate extraction (**2**). The combined fraction weighed 1.65 grams, contained 7.0% nitrogen and had a radioactivity of 1.51×10^6 c.p.m.

Discussion of results

That acetate is readily metabolized by the tomato fruit is apparent from the data obtained. Approximately 45% of the radioactivity of the injected substrate was found in the respiratory CO_2 within 28 hours. Radioactivity was incorporated also in the acid and protein fractions isolated from the juice and pulp (table I).

As shown by the C^{14}-labeling pattern in table II, only the carboxyl groups of citrus and malic acids were found to be labeled. In the case of

TABLE I

RADIOACTIVITY OF FRACTIONS ISOLATED FROM TOMATO FRUITS (483 GM.) FOLLOWING INJECTION WITH C^{14}-LABELED SODIUM ACETATE.

Fraction	Weight in gms.	Radioactivity c.p.m. $\times 10^6$	Per cent. of incorporated radioactivity
Total acids	0.40	2.18	10
Protein	1.65	1.51	7
CO_2	0.57	9.89	45

malic acid, the labeling pattern could be explained on the basis of Krebs cycle operation, whereby the radioactive acetate carboxyl group is introduced into the cycle through citrate formation and eventually appears equally distributed in both carboxyl groups of malic acid. This mechanism, however, could not account for any net synthesis of plant acid, which apparently also is an important process in the fruit, since removal of any of the Krebs cycle acids would immediately discontinue the cyclic process. Consequently, it is necessary that some other means are available for synthesizing C_4 acids. Such synthesis could occur by the WOOD-WERKMAN reaction (**12**), by the "malic fixation" scheme (**4**), or by a tail-to-tail condensation of the THUNBERG type (**9**) which also results in an exclusive carboxyl labeling of the C-4 acids when starting from carboxyl labeled acetate.

TABLE II

DISTRIBUTION OF RADIOACTIVITY IN CITRIC AND MALIC ACIDS FROM TOMATO FRUITS UTILIZING CH_3C^{14}-OOH.

Citric acid	C.p.m. × 10^5 per mM	Malic acid	C.p.m. × 10^5 per mM
Whole molecule	5.67	Whole molecule	3.03
$C_1 + C_5$	4.28*	$C_1 + C_4$	3.02*
C_6	1.39	$C_2 + C_3$	0.00
$C_2 + C_3 + C_4$	0.00	....	....

*C.p.m. activity per 2 mM of carbon.

If citric acid in the tomato fruit is formed by the oxalacetate-acetate condensation via the Krebs cycle, assigning C_4 and C_5 of citric acid to the acetate residue, then C_6 and C_1 of citric acid should correspond to C_1 and C_4 of malic acid, respectively. When the specific activities of these carbon atoms are calculated to equal concentrations in the fruit to eliminate the dilution factor, the values obtained are in close agreement. Thus,

specific activity of C_1 or C_4 of malic acid $= \frac{1}{2}(3.02 \times 10^5)$
$= 1.51 \times 10^5$ c.p.m./mM
specific activity of C_6 of citric acid $= 1.39 \times 10^5$ c.p.m./mM
malic acid content $= 2.65$ mM
citric acid content $= 2.08$ mM
specific activity of citric acid on the same concentration basis $= 1.39 \times 10^5 \times 2.65/2.06$
$= 1.79 \times 10^5$ c.p.m./mM

Similarly, if it is assumed that the specific activities of C_6 and C_1 of citric acid are equal as a result of the randomization process of oxalacetic acid equilibrating with symmetrical C_4 acids prior to the condensation reaction, then the specific activity of C_5 and the ratio C_5/C_1 can be calculated from the data. The observed ratio of 2.08 is in good agreement with the theoretical ratio of 2.0, derived on the basis of OGSTON'S (**3**) concept of the asymmetric nature of citric acid (**4, 5**).

Summary

Carboxyl C^{14}-labeled sodium acetate was injected into the locules of mature green tomatoes. Radicactivity appeared in the respiratory CO_2, which accounted for 45% of the original amount injected. Labeling also occurred in the protein and acid fractions isolated from the juice and tissue. Degradation of malic and citric acids showed only the carboxyl groups to be labeled. The relation of the data to the possibility of Krebs cycle operation in the tomato fruit are discussed.

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A TECHNIQUE FOR REMOVAL OF ASEPTIC CYLINDERS FROM CARROT ROOT

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Received December 8, 1952

This paper outlines a simple procedure for removing aseptic uniform explants from carrot root. The method is similar to that reported by Caplin and Steward (1) but differs in the design of the cutting blades and the method of cannula application.

The cutting blades consist of two parallel Durham-Duplex razor blades rigidly held about 3 mm. apart by interposed pieces of stainless steel. Stainless steel bolts clamp the blades firmly against the steel pieces as shown in figure 1. A handle may be attached at one end to facilitate manipulation. It is noted that the cutting edges of the blades are not superimposed but that in operation one will precede the other by about 5 mm.

In use the broad end of a sterile carrot is vertically impaled on a dissecting needle held erect on a rubber stopper. The carrot is grasped by its broad end so that the dissecting needle aids stability. The cutting edge of the "guillotine" is pressed firmly against the free end of the root so that a cross section will be cut. It is necessary that the blade with the leading cutting edge be located distal to the dissecting needle and the blade with the following edge be located proximal to the needle. A slight simultaneous reciprocal rotation of carrot and guillotine facilitates cutting. The cut is continued only until the lead blade has severed the distal root section. This causes one section of the carrot to drop off while the other remains attached by a small area of tangential tissue. The purpose of one cutting edge preceding the other is to allow the leading blade to completely sever the carrot root, while the following does not. In this way, a symmetrical disc of tissue remains loosely attached to the root which is used as a handle. The cylinder is uniformly thick at all points while its diameter depends on the width of the carrot.

The remainder of the operation consists of removing sterile cylinders from the phloem of the partially severed carrot disc. This is done by inserting an alcohol sterilized cannula parallel to the axis of the root. If the cylinder is to be removed exactly one mm. outside the cambium a small measuring flange may be attached to the cannula near its cutting edge. At a depth of 3 mm. the instrument frees the cylinder which lodges in the barrel of the cannula. The operation is repeated continuously until about six cylinders have been freed and lodged in the cannula. In the barrel of the cannula is a wire plunger which can be manipulated with the thumb so that one plug at a time can be ejected.

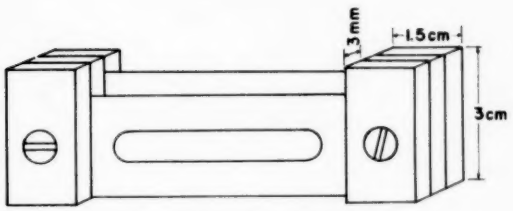

FIG. 1. Cutting apparatus comprising two parallel rigid razor blades held firmly about 3 mm. apart by interposed stainless steel.

An average of one separate placement per minute on test medium to determine freedom from contamination can readily be made. Of 325 inoculations only five plugs revealed contamination at the end of two weeks when grown directly on White's carrot medium containing 15% coconut milk.

By the present method one carrot can provide several hundred aseptic uniform cylinders. The technique is rapid because of the minimum number of operations between isolation and inoculation. The technique can also be used on relatively thick soft stems as well as other roots and tissues.

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PANTOTHENIC ACID AND ACETIC ACID UTILIZATION IN TOMATO FRUIT-SET[1]

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Received December 22, 1952

Fruit-set in the tomato has been shown to be dependent upon the presence of mature leaves (**3**). The nature of the nutritive materials supplied by the leaves has been studied by use of excised tomato flowers cultured on an agar medium to which various nutrients have been added. Of the organic acids tested, all those participating in the Krebs tricarboxylic acid cycle were found to be effective in sustaining fruit-set, but several acids not in the Krebs cycle were essentially ineffective in fruit-set. One of these ineffective acids was acetic acid.

It has been demonstrated that coenzyme A, which contains pantothenic acid as a functional group (**5, 6**) is required for the utilization and metabolism of acetate (**4, 5, 7, 8, 9**). The present report demonstrates that if pantothenic acid is supplied to excised tomato flowers, they are capable of utilizing acetic acid as effectively as acids of the Krebs cycle in fruit-set.

The methods and materials used in these experiments are identical with those reported in the previous study (**2**). Unset flowers of a self-sterile strain of John Baer tomato were excised, sprayed with auxin, and subsequently grown on agar culture media to which the nutrients being tested had been added. The flowers which had set fruit were recorded after 8 days. At least 15 flowers were used for each value reported.

If excised flowers are sprayed with auxin (30 mg./l. *p*-chlorophenoxyacetic acid) and placed on an agar medium containing only acetic acid, very little fruit-set is obtained over a wide concentration range. Such a response is seen in the "control" curve of figure 1. If, however, pantothenic acid (10 mg./l.) is included in each dish, a striking quantitative response to acetic acid is obtained (fig. 1) reaching 60% fruit-set at 10^{-3} M acetic acid. The effectiveness of pantothenic acid in facilitating the acetate utilization is quantitative over a concentration range of 0.01 to 10 mg./l. of pantothenic acid, the last concentration producing the greatest effect.

If the pantothenic acid is facilitating the conversion of acetate to citrate via the reaction catalyzed by coenzyme A, one would expect the effect to be blocked by fluoroacetate. Inclusion of sodium fluoroacetate (10^{-4} M) in each dish with the pantothenic acid did in fact effectively inhibit fruit-set at each level of acetic acid (fig. 1).

If the pantothenic acid is actually facilitating the utilization of acetate, the effect should be specific for that acid. Testing several different organic

[1] Journal paper no. 665; Agricultural Experiment Station, Lafayette, Indiana.

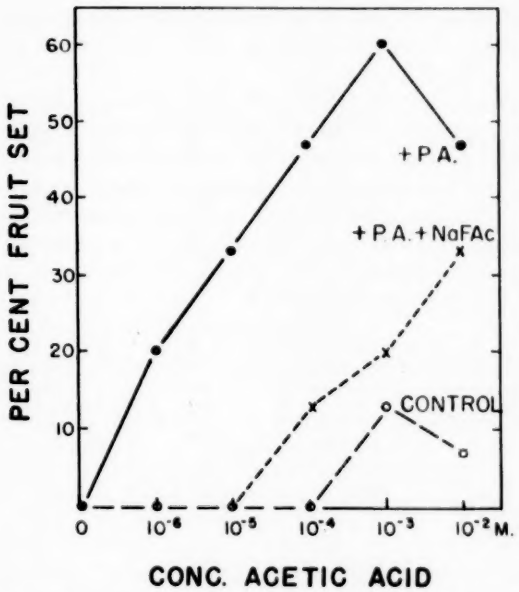

FIG. 1. Effects of various acetic acid concentrations upon fruit-set in excised tomato flowers, comparing acetic acid alone, in the presence of pantothenic acid (10 mg./l.), and in the presence of pantothenic acid plus sodium fluoroacetate (10^{-4} M).

acids for their effectiveness alone and in the presence of pantothenic acid (table I) revealed that only acetic acid utilization in fruit-set is appreciably improved by the presence of the vitamin. Whereas, the vitamin resulted in a 40% improvement in the presence of acetic acid, only a 27% improvement was obtained in the presence of oxalic acid and essentially no change with any of the 5 other acids tested. The pantothenic acid effect, then, appears to be specific for acetic acid of the acids tested.

TABLE I

EFFECTS OF VARIOUS ORGANIC ACIDS UPON FRUIT-SET IN THE PRESENCE OF ADDED PANTOTHENIC ACID.

Acids	Per Cent Fruit-set		
	Acid alone (10^{-3} M)	Acid plus P. A. (10 mg./l.)	Gain due to P. A.
Water control	0	13	13
Acetic acid	13	53	40
Oxalic	13	40	27
Citric	26	33	7
α-ketoglutaric	40	47	7
Succinic	47	47	0
Fumaric	60	53	− 7
Malic	47	40	− 7

If the utilization of acetic acid by tomato flowers is limited by a low pantothenic acid content, one would expect a low content of that vitamin in the ovary. Results of microbiological assay of the pantothenic acid content of ovaries, young set fruits, and leaves of tomatoes are shown in table II. In comparison with other published data on other tomato plants (**9, 10**) the pantothenic acid content of these plants is several-fold lower. Therefore, it appears that this particular variety possesses a relatively low pantothenic acid content. The data in table II show a lower pantothenic acid content in the fertilized ovaries than in unfertilized ovaries or leaves, suggesting that perhaps in the process of fruit-set there is a utilization or conversion of pantothenic acid to an unassayable form.

TABLE II

PANTOTHENIC ACID CONTENT OF SOME JOHN BAER TOMATO TISSUES.

Tissue	Micrograms P.A. per gram fresh weight
Unfertilized ovaries	3.74
Fertilized ovaries	3.71
Leaves	6.34

The experiments reported here indicate that acetic acid cannot be effectively utilized as a nutrient for fruit-set by the excised tomato flowers tested unless pantothenic acid is supplied as well. It is interesting to note that EBERTS *et al.* (**1**) observed no increase in respiration of two types of tomato tissues when acetic acid was supplied as substrate. The other common organic acids which he tested produced respiratory increases which indicated metabolic utilization.

Two implications may be drawn from our data. First, the apparent significance of organic acids of the Krebs tricarboxylic acid cycle in tomato fruit-set suggested previously (**3**) is strengthened by the demonstration that an acid not in the cycle can become effective in fruit-set if entry into the cycle is facilitated. Second, a metabolic pathway was found which was apparently limited in the natural state, and by the supplying of appropriate materials that type of metabolism was forced upon the plant organ and was utilized by it effectively to support a developmental process.

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GROWTH OF TOBACCO SEEDLINGS WITH NITRATE AND ITS REDUCTION PRODUCTS

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Received February 26, 1953

Of the five known products of nitrate reduction only ammonia is considered to be equivalent to nitrate as a source of nitrogen for green plants. Nitrous acid (HNO_2) and hydroxylamine (NH_2OH) have been included in this category by a few investigators (**1, 2**), but are known to be toxic and utilized slowly and with difficulty. The availability of nitrohydroxylaminic acid ($H_2N_2O_3$) and of hyponitrous acid ($H_2N_2O_2$) is unknown. The importance of such information is that it is necessary for an understanding of the pathway of inorganic nitrogen nutrition in the green plant. An economic aspect also exists since nitrite accumulation in pasturage has been held responsible for poisoning of stock on various occasions.

Methods

The material herein presented was obtained in aseptic culture with seedlings of Maryland Medium Broadleaf tobacco. They were grown for four weeks at 25° C and 500 f.c. with 50 ml. of a mineral solution in 200-ml. Pyrex Erlenmeyer flasks. Agar was added to the solution in some of these experiments to test the effects of a solid medium. The methods employed have been described previously (**4**). Cultures were always in duplicate or more.

Results

The data of table I are averages of results obtained in consecutive experiments. The seedlings were grown both in agar and in liquid cultures having slightly acid or almost neutral reactions. Results were quite similar under these conditions at the nutritional levels employed. All series averages were adjusted on a relative basis (**5**) to the same yield at zero nitrogen addition.

Inspection of table I reveals that nitrogen supplied as nitrohydroxylaminic acid was utilized at the 10 p.p.m. level as effectively as nitrogen supplied as nitric acid. Nitrite-nitrogen was only 52.5% as effective; that of hyponitrite, 0%; and of hydroxylamine, 0%. Nitrite N was inferior to that of nitric and nitrohydroxylaminic acids at all levels of supply. Nitrogen of hyponitrous acid and hydroxylamine was not available as sources of nitrogen supply at any of the concentrations tested, though a very slight growth occurred with hyponitrous acid which may well be due to the presence of other nitrogen compounds as impurities. It is clear, however, that hyponitrous acid was non-toxic at the levels used. Hydroxylamine was toxic at the 10 to 50 p.p.m. nitrogen levels.

TABLE I

DRY-WEIGHTS OF TOBACCO SEEDLINGS GROWN WITH NITRATE AND ITS PRODUCTS OF REDUCTION AS SOLE SOURCES OF NITROGEN FOR FOUR WEEKS.

Nitrogen compound	Formula	P.p.m. of nitrogen			
		0	10	25	50
		mg.	*mg.*	*mg.*	*mg.*
Sodium nitrate	$NaNO_3$	2.4	28.1	45.1	54.7
Sodium nitrite	$NaNO_2$	2.4	15.9	22.0	0.5
*Sodium nitrohydroxylaminate	$Na_2N_2O_3$	2.4	28.4	36.4	1.0
*Sodium hyponitrite	$Na_2N_2O_2$	2.4	2.3	2.8	3.3
Hydroxylamine	NH_2OH	2.4	1.8	1.1	0.1

*These compounds were prepared through the courtesy of Dr. Joseph Rosin of Merck and Company, Rahway, New Jersey.

The optimum concentration for utilization of nitrogen varied widely with these compounds. That of nitrate is shown to be well above 50 p.p.m. and is therefore in agreement with current studies in water-culture. The nitrite and nitrohydroxylaminate optima are at about 25 p.p.m. of nitrogen. It should be realized, however, that these specific optima depend on the very limited volume of nutrient solution (50 ml.) used in these experiments. The quantities of nitrogen per plant added at the different levels were 0, 0.5, 1.25, and 2.5 mg. A distinction must also be made between toxicity and availability, two of the factors that determine the optimum.

Discussion

These results would indicate that the pathway of nitrate reduction in the tobacco plant could proceed only through nitrohydroxylaminic acid to ammonia. It is improbable that nitrite so functions in view of its greatly reduced rate of utilization at all concentrations as compared to nitrate. An analytical study of the plant would be desirable from this viewpoint since both compounds respond to the same qualitative tests almost without exception (**3**). No attempts have been made to distinguish between them by previous investigators. The results with hyponitrite and hydroxylamine would indicate that neither participates to any extent in nitrogen nutrition.

This interpretation is based on the view that the relative importance of a chemical reaction in the plant depends primarily on its necessity for maintenance of optimum (maximum) rate of growth and differentiation. Many biochemical reactions have been found to take place in the plant, including the formation and destruction of the compounds under discussion. Very little data, however, are available for evaluating the relative importance of such reactions in the life of a plant. Such information can be obtained at present in only one way. Namely by demonstrating that the biochemical compounds concerned must always be present, and that they are produced and used at a rate sufficient to maintain optimum growth and differentiation of the plant. Only nitrate, ammonium, and probably nitro-

hydroxylaminate meet or can meet these requirements in the case of the known inorganic nitrogen nutrients for the green plant. The fallacy of accepting all biochemical reactions in the plant as of primary importance is evidenced by the ability of the plant to alter purely synthetic compounds never present under natural conditions.

Further experimental proof of the validity of these interpretations could be obtained by the use of very large volumes of nutrient solution or the use of rapidly flowing "drip cultures." A concentration of 10 p.p.m. of nitrogen as the sodium salt of nitrohydroxylaminic acid should give plants having the same rate of growth and final maximum yield as the same or higher concentrations of nitrate-nitrogen. A similar result would be unobtainable with nitrogen as nitrite, hyponitrite or hydroxylamine at any concentration. It should not be overlooked, that because of the limited volumes of nutrient solution, the concentrations of nitrate recommended for greenhouse water-cultures are far above those usually available in the soil solution.

The conclusions drawn on the basis of this study with the tobacco plant would appear to confirm those previously reached in a similar study with a fungus (**3**). These are that nitrohydroxylaminic acid participates as an intermediate in nitrate reduction to ammonia, and that hyponitrous acid, hydroxylamine, and probably nitrous acid do not.

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RESPONSES OF PLANTS TO RHENIUM

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Received April 22, 1953

Preliminary studies of the effects of rhenium salts on plants were initiated by the junior author at Harvard University during 1946 and 1947 under the direction of Professor K. V. Thimann. The interest in this metal was stimulated by his results in an earlier project testing physiological effects of various unusual elements for which such data were not as yet available. In this project, which included elements such as tellurium, gallium, gold, etc., the effects of rhenium were found to be unique. The symptoms produced were striking, and they were produced at very low concentrations. The information obtained was made available to the senior author who in turn supervised a series of student projects bearing on this problem as part of his courses in plant physiology. Confirmation of the original observations was obtained, and the investigations were expanded to probe the mechanism involved.

The element rhenium (Re), atomic number 75 and atomic weight 186.31, is classified with manganese, technetium, and neptunium in group VII-A of the periodic system of the elements. As a metal, rhenium is interesting because of its high melting point ($3440 \pm 40°$ C). The element is unique in its reported ability to achieve all possible valence states from -1 to $+7$. Metallic rhenium is readily available at a cost of about $3.60 per gm.

Solutions were prepared by dissolving a known weight of metallic rhenium in concentrated HNO_3, adjusting to the desired pH (pH 6 in recent work) with NaOH, and diluting to desired strength with distilled water. At higher pH values precipitation occurs. It was also found that solutions must be reasonably fresh to give consistent results since a continual, gradual crystallizing out occurs even in the lower pH range.

Several different procedures for introducing rhenium into the plants were used, all of which proved effective. These included (1) a foliar spray with a nasal atomizer, (2) subepidermal injection with a hypodermic syringe, (3) direct addition to the culture solution surrounding the roots, and (4) direct addition to the soil. Solutions containing from 0.1 to 200 mg. Re/l. have been employed.

The nature of the responses varied among different plants. In a series of experiments in which rhenium solutions were applied to sand cultures, it was found that concentrations of 1 mg./l. or less produced no observable effects in all experiments. Concentrations between 5 and 50 mg./l. (2.68×10^{-5} to 2.68×10^{-4} M) resulted in darkening of the green coloration and in in-

creasingly severe morphological modifications. Concentrations above 50 mg./l. generally proved lethal. In the monocots corn and wheat, the coleoptiles of the seedlings appeared to toughen and thicken so that the stem did not break through in the normal manner. Twisting and malformation of seedlings together with apparent fusion of the sheaths of the young leaves to the stem resulted. The general appearance in advanced cases was one of hypertrophic expansion of growing portions. If treated for a time just sufficient for development of symptoms, then returned to normal conditions, the plants recovered. The treatment and recovery could be repeated several times. Young dicotyledonous plants, such as tomato and pea, exhibited the same increased darkening of the green coloration together with progressively increasing scattering of small brownish spots which were more abundant toward the margins of the blades. In the higher and more toxic concentrations, the leaflets cupped under, the leaves curved backward, and necrosis developed in the young leaves and terminal leaflets of mature leaves and progressed downward through the plant. Symptoms developed in tomato plants within two days, while death occurred within five days, in tomato plants exposed to 100 mg./l. spray. Plants which have responded to spray applications of 50 mg. Re/l. include tomato, bean, pea, corn, wheat, oat, geranium, and coleus.

Exploratory experiments were undertaken to investigate the mechanism of toxicity. Among these were the similarity between rhenium toxicity and those of manganese and phosphorus, the effects of the element on cell division, and its effects on respiration. Young soil-grown tomato plants were transferred to mineral solution culture and allowed to recover for a period of a week. At the end of this period, manganese chloride was added to the solutions so as to give a series of experimental plants exposed to concentrations of manganese including 0.0, 1.0, 10, 100, 500, 1000, and 2500 mg./l. Simultaneously, comparable plants were similarly treated with 50 mg. of rhenium per liter. At none of the concentrations of manganese were the symptoms similar to or nearly so marked as those developed in 50 mg. Re/l. The threshold of toxicity was at a lower concentration in the case of rhenium ($\pm 2.68 \times 10^{-4}$ M) than for manganese ($\pm 1.82 \times 10^{-2}$ M). Experiments probing the toxic symptoms of phosphorus have been inconclusive because of the difficulty in maintaining toxic concentrations of phosphates in these calcium-containing solutions. Deficiency symptoms of phosphorus were not found to be identical to those produced at a toxic concentration of rhenium. An idea of of the dosage required to produce symptoms was gained by a study of sand cultures of tomato. Plants were watered daily and in the watering received a supplement of 25 ml. of complete nutrient solution. Experiments were begun on plants 7 to 8 inches tall by supplying them with 1.25 to 2.50 mg. of rhenium per day in addition to the usual nutrient supplement. On the 7th day, after a total dosage of 11.25 mg./plant, symptoms began to develop.

The effects of rhenium on cell division were studied by using cultures of the alga *Coelastrum* (Yale strain) grown in Chu 10 medium (1) containing

different concentrations of rhenium. Growth was followed spectrophotometrically over a three-week period. Concentrations of 1 mg./l. or less showed no reduction in growth when compared to controls. Between 5 and 50 mg./l. an increasing reduction in optical density was found. Nearly total inhibition of growth occurred at 80 mg./l. These values correspond quite closely with concentrations found effective in the angiosperms previously tested. This effect of rhenium on the rate of cell division in *Coelastrum* stimulated a cytological study. Well-developed onion roots were exposed to 50 mg./l. solutions of rhenium for 24-, 48-, and 72-hour periods, after which the root tips were fixed in Carnoy's fixative (3:1) and smeared in aceto-carmine. No irregularities in chromosome morphology or mitotic figures were detected when examined under magnifications up to 1250×.

The effect of rhenium on respiration was studied using *Chlorella* (Yale strain) grown in Maerten's solution and employing the Warburg technique at 25° C. The formulation for Maerten's solution was provided by Dr. Ralph Lewis and Dr. Joyce Lewin, now of Halifax, Nova Scotia. In the run, oxygen uptake was followed for a period of an hour prior to addition of rhenium from the sidearm, then followed for an additional hour. Concentrations of 1.0 and 80 mg. Re/l. produced no detectable changes in rate of gas exchange. In order to check these results, and to eliminate possible interference by constituents of the culture solutions previously used, a comparable experiment was run using sections of potato tuber. These sections from a fresh potato were 10 mm. in diameter and 75 μ thick, and were washed in running tap water for 24 hours prior to experimentation. Concentrations including 1, 50, and 100 mg. Re/l. were employed. No important differences in oxygen uptake were detected during the 1½ hours subsequent to mixing, although one manometer registered a marked decrease in oxygen uptake during the run. Since in this earlier experiment an indication of possible decline in rate of respiration was noted, a run was made to determine changes in the rate over longer periods of time. A concentration of 100 mg. Re/l. was used in the experimental flasks, and the time of exposure of potato tuber slices to rhenium was increased to five hours. Whereas no effect was detected during the first hour, consistent with the previous data, during the longer five-hour run a gradual decline in respiration was observed. After five hours, the oxygen uptake had dropped from 0.07 μl. O_2/mg. dry weight × hr. to 0.04 μl. O_2/mg. dry weight × hr., an inhibition of approximately 43% in five hours. The control exhibited no decline in rate of oxygen uptake during this period. From these studies, rhenium appears to have a pronounced but delayed depressing effect on the rate of respiration.

The basis of the marked darkening of green coloration was studied by extracting normal and symptom-exhibiting leaves with 80% methanol. The absorption spectra obtained with a Coleman Universal Spectrophotometer were practically identical. Thus, the color change was apparently not the result of alterations in the nature or concentration of the chloroplast pigments. Extraction for non-chloroplast pigments has not as yet been undertaken.

Summary

Summarizing the above information, marked response by plants to rhenium is noted. The symptoms are severe and develop fairly rapidly. Concentrations from 5 to 50 mg./l. produced increasingly intense symptoms, while those in excess of 50 mg./l. are generally lethal. The total dosage required to produce symptoms in tomato was approximately 11 mg. Darkening of the green coloration is apparently not due to changes in chloroplast pigments. The symptoms are not the same as those of manganese toxicity, and apparently differ from those of phosphorus deficiency. Cell division is reduced, but no obvious modifications in chromosome morphology or mitotic figures were detected. Respiration is inhibited, the inhibition being gradual and detected only after several hours of exposure to toxic concentrations. The mechanism of operation is not yet known, but the effects of rhenium on rate of cell division and rate of respiration suggest avenues of approach to the problem.

The authors gratefully acknowledge the assistance of Messrs. H. Rogelio Cuellar, Charles Dame, Clinton K. Davis, Jr., Herbert T. DeRigo, John A. Jagschitz, Charles H. Lamoureux, Charles Mason, and Robert E. Partyka. For the cytological work, the authors are especially indebted to Prof. Robert Lepper, Jr., Head of the Botany Department.

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NOTES

Dean Walter F. Loehwing of the State University of Iowa served as secretary of the Editorial Board from 1939 to 1946, when he became Editor-in-chief, of PLANT PHYSIOLOGY. He continued as Editor-in-chief through the July issue of 1953. His contributions to PLANT PHYSIOLOGY, to the Journal, and Society have been very great. The present editor feels that he speaks for all of the present and former officers of the Society, members of the Editorial Board, and members at large when he conveys in this space our thanks to Dean Loehwing for his years of devoted service to our Journal. This editor appreciates Dean Loehwing's helpfulness and courtesy in transferring the Journal into new hands.

Dr. Robert S. Muir, of the same university, has served as Secretary of the Editorial Board since 1951. His share in the work of editing PLANT PHYSIOLOGY since 1951 has been heavy. Fortunately, he continues as a member of the Editorial Board as an associate editor. He has been of real aid to the new editor.

The new members of the Editorial Board elected last spring, and serving until 1956 are Doctors D. I. Arnon and R. H. Burris. The Executive Committee appointed the following: Doctors Allan H. Brown to serve until 1958, Perry Stout, 1957, and Bernard Meyer, 1957. These men are all welcome additions to the Editorial Board.

American Society of Plant Physiologists, *Annual Meeting, Madison, Wisconsin, September 1953:*—Three of the Society's committees were disbanded upon recommendation of their chairmen. These were the "Committee on Interrelations of the American Society of Plant Physiologists and the Physiological Section of the Botanical Society of America" headed by Dr. B. S. Meyer, "The Committee on Chemical Methods" headed by Dr. A. J. Haagen-Smit, and "The Committee on Aid in Preparation of a Handbook of Biological Data," headed by Dr. David Goddard.

The Executive Committee approved the proposal that the term of office of the President, Vice-President and Secretary begin on January 1 following the usual spring election. It was also decided that the Society would meet with the A.I.B.S. at the University of Connecticut in 1956. Dr. K. V. Thimann was appointed to the Board of Trustees, Dr. P. J. Kramer was appointed as the Society's representative to the Governing Board of the A.I.B.S., and Dr. J. Fisher Stanfield was reappointed as Executive Secretary Treasurer of the Society.

Dr. James Bonner reported that a brochure for vocational guidance in the field of plant physiology has been prepared by his committee and will shortly be ready for submission to the Society for publication. The Society has had a great many requests for literature of the type prepared from both high school and college advisers. Following publication, it is tenta-

tively planned to have copies available through the Executive Secretary's office.

Early submission of Dr. van Overbeek's Committee report on "Nomenclature of Plant Growth Substances" for publication was urged.

The Charles Reid Barnes Award for 1953 was given to Dr. Henrik Lundegardh who received it in person. Dr. R. N. Robertson, Noburo Kamiya, Georg Melchers, and Richard Harder were honored through election to be Corresponding Members of the Society.

The Executive Secretary-Treasurer's Annual Report indicated that the Society is in sound financial condition and is continuing to grow steadily. Following is a condensation of his report.

Membership: Active members, July 1, 1952, 1007; July 1, 1953, 1047, a net gain of 40 members for the year. The membership now includes 32 life members, 18 corresponding members, 7 institutional members, 24 Canadian members, 873 U.S. members, and 97 foreign members.

Subscriptions: Vol. 27 in U.S., 375; Foreign, 617; Total for Vol. 27, 992. Vol. 28 in U.S., 375; Foreign, 675; Total for Volume 28, 1050, representing a net gain of 58 subscribers during the year. The number of paid copies of Plant Physiology mailed to regular members and subscribers for 1953 totaled 2097.

Summary of Cash Accounts:

Checking Account, July 1, 1952	$ 9,254.08
Savings Account, July 1, 1952	6,000.00
Cash Balance, July 1, 1952	$15,254.08
Receipts, 1952–1953	21,611.82
Total Cash, 1952–1953	$36,865.90
Expenditures, 1952–1953	18,887.70
Cash Balance, July 1, 1953	$17,978.20
Cash Balance, July 1, 1952	15,254.08
Excess Receipts over Disbursements, 1952–1953	$ 2,724.12

Summary of Assets:

	July 1, 1952	July 1, 1953
Checking Account	$ 9,254.08	$11,978.20
Savings Account	6,000.00	6,000.00
Barnes Endowment Fund	4,000.00	4,000.00
Hales Endowment Fund	2,500.00	2,500.00
General Endowment Fund	16,700.00	18,700.00
Total Assets of the Society	$38,454.00	$43,178.20

The new editor of our Journal, Dr. David Goddard, submitted a report in which it was stressed that measures must be taken immediately to speed publication of the steadily increasing flow of manuscripts as well as to eliminate the large back-log of papers now awaiting publication. Following discussion growing from this report and that of Dr. K. V. Thimann, Chairman, "The Committee to Study Publications," it was noted without dissent that beginning with the volume for 1954 the number of issues of *Plant Physiology* per year shall be increased to six. A budget was sub-

mitted by Dr. Goddard to cover costs of publication and delivery of our journal for 1954. After careful consideration $18,500.00 was voted for these purposes: $16,500.00 for printing, $1,000.00 for mailing and $1,000.00 for editorial office expenses. In view of the increase in size of the journal it was voted that its cost to subscribers be increased to $14.00 per year beginning with volume 29. It is estimated this will increase the Society's income sufficiently to make it unnecessary to use capital funds for publication.

Three new members were elected to the Editorial Board by the Executive Committee: Dr. Allan H. Brown, 1958, Dr. Perry Stout, 1957, and Dr. B. S. Meyer, 1957.

A good demand for complete sets of *Plant Physiology* exists. Because the first five volumes are lacking this demand cannot be met. A publisher, J. Walter Johnson, asked consent of the Society to permit it to reprint these volumes. No subsidy by the Society was desired. Consent was given.

The final act of the Executive Committee was not to authorize the Executive Secretary to proceed with preparation and publication of a new directory for the Society. Respectfully submitted, Aubrey W. Naylor, Secretary.

Hendrik Lundegårdh was elected Charles Reid Barnes life member.

Henrik Lundegårdh was born October 23, 1888 in Stockholm. He received his scientific training in the University of that city and was appointed assistant professor (docent) in the University in 1912. His early research was on nuclear division and the structure of protoplasm, but his interests gradually shifted toward physiology, especially tropisms. In 1916 he moved to the University of Lund where he continued his studies on geotropism in roots and shoots and also studied phototropism. In 1917 Lundegårdh founded an ecological station where he and his students began work in experimental ecology. This work resulted in what is probably his best known book, Klima und Boden, which appeared in 1925, and in an English edition in 1931.

In 1926 Lundegårdh was appointed professor and head of the Botanical Division of the Central Agricultural Experiment Institute at Stockholm. Here he was able to apply physiological and ecological principles to various agricultural problems. He gave especial attention to the interaction of various factors on crop production and made extensive studies of the temperature optimum of photosynthesis and the manner in which it is affected by various environmental factors. It was here that Lundegårdh began the studies on salt absorption by plants for which he is best known. In connection with these studies he developed methods of spectrographic analysis to a high level of efficiency.

Lundegårdh moved to the Institute of Plant Physiology of the newly created Royal Agricultural College at Uppsala in 1935 where he is now professor and director. Here he has continued his work on salt absorption and

related problems such as the relation between respiration and salt accumulation, the role of the cytochrome-cytochrome oxidase system in salt accumulation, the mechanism of root pressure, and root growth. He has also maintained his interest in ecological physiology and such problems as sap movement in trees. Much of this work is done in a private laboratory on the shores of the Baltic Sea.

As a result of his work on plant nutrition he published his work on analytical methods, Die Blattanalyse, in 1944, and in 1950 a textbook of plant physiology and anatomy. In addition to his scientific research Lundegårdh has found time to publish articles of a philosophical nature and to cultivate his musical talents.

GROWTH AND DIFFERENTIATION IN PLANTS: *A Monograph of the American Society of Plant Physiologists;* Edited by W. E. Loomis.

A thorough discussion of the morphology, physiology and chemistry of growth and reproduction. Emphasis on recent developments in hormones, hormone balance, photoperiodism, protein metabolism, and growth correlation including electrical correlation.

Eighteen chapters by twenty-three authors as follows:

1. Growth and differentiation—an introduction and summary.—W. E. Loomis, Iowa State College.
2. Plant morphogenesis.—E. W. Sinnott, Yale University.
3. Growth of the root tip.—Gabriel Baldovinos, Department of Agriculture, Mexico City, D. F.
4. Elongation of the cotton fiber.—J. C. O'Kelley, University of Alabama, and P. H. Carr, Iowa State College.
5. Anatomical differentiation in root and shoot axes.—Katherine Esau, University of California.
6. Some elementary mathematics of plant growth.—R. E. Buchanan, Iowa State College.
7. Heterosis.—G. F. Sprague, USDA and Iowa State College.
8. Dormancy.—N. C. Thornton, La Lima, Honduras.
9. Reactions of plants to photoperiod.—A. W. Naylor, Duke University.
10. Vernalization of growing plants.—H. C. Thompson, Cornell University.
11. Growth correlation.—W. E. Loomis, Iowa State College.
12. Bioelectric fields and correlation in plants.—Hilda F. Rosene and E. J. Lund. Austin, Texas.
13. The auxins.—Solon A. Gordon, Argonne National Laboratory, Chicago, Illinois.
14. The hormonal control of flower initiation.—James Bonner and James Liverman, California Institute of Technology, Pasadena, California.
15. Structure and synthesis of protoplasm.—H. S. McKee, Com. Res. Sci. Org., Sydney, Australia.
16. Cellular differentiation: an experimental approach.—Barry Commoner, and Milton L. Zucker, Washington University, St. Louis, Missouri.
17. Some factors associated with diseased growth in plants.—A. J. Riker and A. C. Hildebrandt, University of Wisconsin.
18. Comparative physiology of heterotrophic growth in plants.—S. H. Hutner, Haskins Labs., New York City.

The price will be **$7.50**, but if society members order promptly from J. Fisher Stanfield, and accompany their order with a check, they may obtain the book for **$5.50**.

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS

Organized in 1924 for the advancement of Plant Physiology as a pure and applied field of botanical science

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OFFICERS FOR 1952–1953

President: Dr. Daniel I. Arnon,
University of California, Berkeley, California

Vice-President: Dr. Folke Skoog,
University of Wisconsin, Madison, Wisconsin

Secretary: Dr. Alden S. Crafts,
University of California, Davis, California

Executive Secretary-Treasurer: Dr. J. Fisher Stanfield,
Miami University, Oxford, Ohio

Any one actively interested in Plant Physiology is eligible for membership in the Society. Candidates for membership must be recommended by two members, and approved by executive committee, or by the vote of the Society at some annual or special meeting.

The payment of the annual dues to the Society entitles the members to receive the official journal, Plant Physiology. They will receive in addition the regular bulletins and announcements sent out periodically by the secretary. Members are invited to take a personal interest in the growth and development of the Society.

Manuscripts for Plant Physiology should be addressed to Prof. David R. Goddard, Botanical Laboratories, University of Pennsylvania, Philadelphia, Pennsylvania. Drawings and photographs should not exceed 8.5 × 11 inches in size, should be numbered correctly on the back, and separate typewritten legends included with the text of the manuscript. Tables and cuts should be used sparingly, and the text presented in concise form. Instructions to authors for preparation of manuscripts are given in the January number, Volume 28, 1953, of Plant Physiology, 149–150.

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